

University of Alberta

Heat, acid, and salt tolerance of leucocin-resistant *Listeria monocytogenes*

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Master of Science

in

Food Science and Technology

Department of Agricultural, Food, and Nutritional Science

Edmonton, Alberta

Fall 2005



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DEDICATION

In loving memory of Ying Tip Mah.

ABSTRACT

The stress tolerance of wild type and leucocin-resistant (leu^R) *Listeria monocytogenes* ATCC 15313, CDC 7762, and FS 15 was studied in high temperature (60°C), low pH (acetic acid, $\text{pH} = 3.6$), and saline (8% NaCl) conditions. Heat tolerance of leu^R variants of *L. monocytogenes* was the same as the wild type strains, except for one leu^R variant of *L. monocytogenes* FS 15 (FS15L2) that had increased thermotolerance. A decrease in acid tolerance was observed for leu^R variants of *L. monocytogenes* ATCC 15313 and CDC 7762, but the acid tolerance of leu^R *L. monocytogenes* FS 15 was the same as that of the wild type strain. There was no difference in the salt tolerance of wild type and leu^R *L. monocytogenes*. Restriction fragment differential display-PCR and confirmatory dot blot analysis did not identify any differentially expressed genes.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Lynn McMullen for her help and guidance throughout my graduate studies. Her assistance and support have been greatly appreciated.

I would also like to thank my supervisory committee member Dr. David Bressler and my examining committee member Dr. Susan Jensen for all their contributions to this thesis.

Special gratitude is reserved for Susan Gibson, Dr. Rodrigo Bibiloni, Dr. Marco van Belkum, and Dr. Lucas Gursky for providing invaluable technical support and suggestions for my project.

I want to thank my fellow graduate students in the food microbiology lab and all my friends in AFNS for offering input on my experiments, for their friendship, and for helping to make every day on campus a truly enjoyable experience.

Finally, I would like to say thank you to my friends and family, especially my parents, for their love and support. Without them, I would not be where I am today.

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ABBREVIATIONS

APT	all purpose Tween
ATP	adenosine triphosphate
AU	arbitrary units
BLAST	basic local alignment search tool
CNS	central nervous system
DCCD	<i>N, N'</i> -dicyclohexylcarbodiimide
DEPC	diethyl pyrocarbonate
dNTP	deoxynucleoside triphosphate
EtBr	ethidium bromide
EDTA	ethylenediaminetetraacetic acid
GAD	glutamate decarboxylase
Km	kanamycin
LAB	lactic acid bacteria
LB	Luria-Bertani medium
leu ^R	leucocin A-resistant
LLO	listeriolysin O
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	national center for biotechnology information
PCR	polymerase chain reaction
PTS	phosphotransferase system
RAPD	randomly amplified polymorphic DNA
RFDD-PCR	restriction fragment differential display PCR
RT-PCR	reverse transcriptase-PCR
TBE	tris-borate-EDTA
TFA	trifluoroacetic acid
TSAYE	tryptic soy agar with 0.6% yeast extract
TSBYE	tryptic soy broth with 0.6% yeast extract
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

1 Introduction

For food safety, bacterial pathogens are a concern because their presence in foods poses a health risk to consumers. One such pathogen is *Listeria monocytogenes*, which can cause mild gastroenteritis and fever in healthy individuals (Hof 2001), but can be deadly to those with compromised or underdeveloped immune systems (Rocourt 1996, Vázquez-Boland *et al.* 2001). Additional characteristics of *L. monocytogenes* make this organism of particular concern to the food industry. Unlike most other foodborne pathogens, not only is this bacterium able to tolerate some of the processing conditions used to kill or inhibit pathogens in foods (Hill *et al.* 2002), but *L. monocytogenes* can also grow during storage at refrigeration temperatures (Junttila *et al.* 1988). Therefore, additional methods have been proposed for controlling this pathogen in foods, such as the use of bacteriocins.

Certain bacteriocins such as leucocin A are considered *Listeria*-active because they have bacteriostatic or bacteriocidal activity against *L. monocytogenes*. However, the potential for controlling *L. monocytogenes* using leucocin alone is limited, in part due to the ability of the organism to acquire resistance to the bacteriocin. The resistance of *L. monocytogenes* to leucocin A and related bacteriocins is well documented, and the resistance mechanisms are being determined (Gravesen *et al.* 2002a, Gravesen *et al.* 2002b, Ramnath *et al.* 2004), but there are conflicting data concerning other phenotypic changes that accompany bacteriocin resistance. One investigation reported no change in the ability of bacteriocin-resistant *L. monocytogenes* to tolerate environmental stresses such as low pH, low temperature, and high salt concentrations (Gravesen *et al.* 2002a). However, previous studies in this laboratory have indicated that the development of bacteriocin resistance in *L. monocytogenes* may result in increased ability to survive exposure to environmental stress (Gao 2001, Aguilar 2004). In particular, Aguilar (2004) determined that a strain-specific characteristic of leucocin-resistant *L. monocytogenes* was higher tolerance to environmental stress as compared with the wild type strain.

The stress tolerance of bacteriocin-resistant bacteria has consequences for the food industry in terms of food safety. If a bacteriocin-resistant strain of a pathogen was to develop an increased ability to tolerate stresses, it might be able to survive the processing conditions that are normally used to kill pathogens in food products. In such a case, previous exposure of the bacterium to a bacteriocin would pose a health hazard rather than improving the safety of the food. Conversely, if acquisition of bacteriocin resistance resulted in a decreased stress tolerance, then bacteriocin use would have the added advantage of decreasing pathogen survivability, independent of the antimicrobial properties of the bacteriocin.

The objective of this study was to determine if leucocin-resistant (leu^R) variants of *L. monocytogenes* had an increased tolerance to environmental stress as compared with the wild type strains. Three strains of *L. monocytogenes* were chosen for study, and three leu^R variants were isolated for each of the strains after exposure to the bacteriocin leucocin A. The strains used were *L. monocytogenes* ATCC 15313, CDC 7762, and FS 15. Strain ATCC 15313 was isolated from rabbits (Murray *et al.* 1926), and is the American Type Culture Collection's type strain of the species. Strain CDC 7762 is a strain from a 1998 outbreak of foodborne disease involving hot dogs (Anonymous 1998). Strain FS 15 was isolated from retail beef wieners in this laboratory, and exhibits unusually high heat tolerance (McMullen, unpublished data). The survival of cells in the presence of environmental stresses (temperature, 60°C; low pH, 3.6; and high salt concentration, 8%) was determined by D-value, or the time required for the bacterial population to decrease by one log unit. D-values were compared among the wild type strains and their leu^R variants to detect increases or decreases in stress tolerance.

To further investigate stress tolerance in the leu^R variants, and to identify which genes were responsible for changes in stress tolerance, gene expression was analyzed using restriction fragment differential display PCR (RFDD-PCR). This technique has been used previously to screen for changes in gene expression in a pediocin-resistant strain of *L. monocytogenes* (Gravesen *et al.* 2000), and it has advantages over alternative screening methods such as subtractive hybridization and subtractive display (Duguid *et al.* 1988, Ariazi and Gould 1996). Restriction fragment differential display PCR allows simultaneous identification of upregulated and downregulated genes, and multiple RNA

samples can be visualized and compared easily on a sequencing gel (Lowe 2000). Furthermore, RFDD-PCR requires much less mRNA than subtractive techniques (Lowe 2000), it is considered to be reproducible, and it provides sufficient coverage to analyze expression of all genes in a given bacterial species (Gravesen *et al.* 2000).

2 Literature Review

2.1 *Listeria monocytogenes*

The bacterium *Listeria monocytogenes* is a Gram-positive rod that was first described in 1926 (Murray *et al.* 1926). Although it was first suspected to cause disease in rabbits, *L. monocytogenes* is now known as an important pathogen that can affect humans and animals. Direct transmission between infected individuals is thought to be rare, and it is considered to be primarily a foodborne pathogen (Janda and Abbott 1999). It is a facultative anaerobe that has been found to grow at temperatures as low as 1.1°C (Junttila *et al.* 1988), with optimal growth occurring between 30 to 37°C (Janda and Abbott 1999). This organism is also very tolerant to environmental stresses such as heat (Farber *et al.* 1988), high salt concentrations (McClure *et al.* 1989), and extremes in pH (Cheroutre-Vialette *et al.* 1997).

The distribution of *L. monocytogenes* in the environment is widespread, and it has been isolated from soil and plants (Weis and Seeliger 1975). The organism has been isolated from both wild and domesticated animals (Murray *et al.* 1926, Blenden *et al.* 1987), and it can also be found widespread throughout households (Beumer *et al.* 1996). In their review on *L. monocytogenes*, Farber and Peterkin (1991) suggest that the organism may even be part of the natural flora in the human intestinal tract, since between 5 and 10% of the healthy population may be asymptomatic carriers of the bacterium. Several large outbreaks of listeriosis in the 1980's were responsible for increasing awareness of the organism's importance in North America (Farber and Peterkin 1991). Of the foods implicated in outbreaks, the most common are ready-to-eat foods (Dalton *et al.* 1997, Anonymous 2002, Frye *et al.* 2002).

2.1.1 Listeriosis

Listeriosis is the disease caused by *L. monocytogenes*. A closely related bacterium, *L. ivanovii*, is also capable of causing listeriosis, but in sheep and cattle (Ramage *et al.* 1999). In humans, healthy individuals rarely get the disease, and most

cases of listeriosis involve hosts that are more susceptible to infection. These high risk groups were summarized well by Rocourt (1996) and they are mostly linked with people that have deficiencies in the part of the immune system that is mediated by T-cells. Pregnant women and neonates are particularly vulnerable, as are the elderly. Some cancers are known risk factors for listeriosis, as are the immunosuppressive therapies for cancer and organ transplants (Rocourt 1996). In 5 to 20% of non-pregnant adults, AIDS is also a predisposing factor for listeriosis, although it is not a common AIDS-related infection (Jurado *et al.* 1993, Vázquez-Boland *et al.* 2001). That may be due to a number of factors such as regular antibiotic treatments and a strict diet which does not include foods commonly associated with *L. monocytogenes* (Rocourt 1996). In addition, HIV infection does not compromise innate immunity or the CD8⁺ subset of T-cells that are responsible for combating infection by *Listeria* spp. (Harty *et al.* 1996, Vázquez-Boland *et al.* 2001). In spite of this, the risk of listeriosis is 65 to 145 times higher in AIDS patients compared with the general population (Jurado *et al.* 1993). There are also some apparent risk factors that are unexplained due to their lack of connection with the immune system. These include chronic conditions such as congestive heart failure, cirrhosis, and alcoholism (McLauchlin 1990c, Rocourt 1996).

The symptoms and severity of listeriosis vary with the age of the host and whether or not a pregnancy is involved. An expectant mother with listeriosis will often be asymptomatic, but may have mild flu-like symptoms such as fever, headaches, and vomiting (Evans *et al.* 1985, McLauchlin 1990b). Mothers rarely experience the more severe symptoms of listeriosis, and usually only if they have other underlying conditions that increase their risk of infection (Fan *et al.* 1989, McLauchlin 1990b). Regardless of whether or not the mother displays symptoms, the *L. monocytogenes* infection may also be passed on to the fetus, resulting in early-onset neonatal listeriosis (McLauchlin 1990b). However, there have also been cases of healthy babies being delivered by mothers with listeriosis (McLauchlin 1990b). In early-onset listeriosis, the fetus can develop pneumonia, respiratory distress, and septicemia, resulting in systemic infection that causes purulent rashes and lesions on the body (Evans *et al.* 1985, Bortolussi and Schlech 1995). The possible outcomes of early-onset listeriosis are abortion, stillbirth, and pre-term labour (Evans *et al.* 1985, McLauchlin 1990b). If infection of the newborn

occurs during or after birth, late-onset neonatal listeriosis will occur (McLauchlin 1990b). Meningitis is common, and can be accompanied by fever, gastroenteritis, and pneumonia (Evans *et al.* 1985, McLauchlin 1990b, Hof *et al.* 2000). In their review, Farber and Peterkin (1991) estimate that the mortality rates for early- and late-onset listeriosis range from 15 to 50% and 10 to 20%, respectively.

In non-pregnant adults, listeriosis usually presents as an infection of the central nervous system (McLauchlin 1990c). As a result of colonization, inflammation occurs in the brain (encephalitis) and meninges (meningitis). Symptoms include changes in consciousness and moving disorders, such as the circling disease exhibited by infected sheep (Blenden *et al.* 1987, Vázquez-Boland *et al.* 2001). Mortality rates for CNS infections have been estimated to range from 20 to 60% (Vázquez-Boland *et al.* 2001). The other common form of listeriosis is septicemia, which has a mortality rate as high as 70% (Vázquez-Boland *et al.* 2001). There are also less typical forms of listeriosis such as endocarditis, pneumonia, jaundice, and localized abscesses in humans, and mastitis in cows (Blenden *et al.* 1987, McLauchlin 1990c). A special cutaneous type of listeriosis can also occur, but it results from direct contact with contaminated animal parts, such as the genitals and placenta of a cow after a listeriosis-induced miscarriage (Cain and McCann 1986).

Classically, descriptions of listeriosis have mainly involved CNS infections, septicemia and bacteremia, and perinatal listeriosis. But due to evidence that *L. monocytogenes* can also cause gastroenteritis in adult humans (Dalton *et al.* 1997, Aureli *et al.* 2000, Hof 2001), the traditional forms of listeriosis are usually referred to in the literature as "invasive listeriosis". The gastroenteritis caused by *L. monocytogenes* is generally self-limiting and is characterized by nausea, vomiting, abdominal cramps, diarrhea, and fever. However, the possibility exists that febrile gastroenteritis can precede invasive listeriosis, so the mild symptoms may have been overlooked in light of the development of a more serious infection. This may explain why *L. monocytogenes* has not been associated with gastroenteritis until relatively recently (Hof 2001).

In general, listeriosis is a serious disease, and mortality rates in humans are around 20 to 30% on average (Vázquez-Boland *et al.* 2001), but cases of perinatal listeriosis have an average rate of 36% (Farber and Peterkin 1991). Because of the

severity of this illness, steps should be taken to minimize exposure of susceptible populations to *L. monocytogenes*.

2.1.2 Pathogenesis

The ability of *L. monocytogenes* to cause disease varies among strains, and it is likely that only a certain subset of strains are virulent in humans (Jacquet *et al.* 2004). Of the 13 serovars of *L. monocytogenes*, serovars 4b, 1/2a, 1/2b, and 1/2c are involved in over 90% of human listeriosis cases (McLauchlin 1990a). There are striking differences in the distribution of serovars between clinical and food isolates. Serovar 4b is responsible for approximately 60% of clinical listeriosis, but serovars 1/2a, 1/2b, and 1/2c combined only make up 32% of cases (McLauchlin 1997). In contrast, when *L. monocytogenes* is isolated from foods, serovars 4b, 1/2a, 1/2b, and 1/2c comprise 22, 32, 15, and 21% of isolates, respectively (McLauchlin 1997). Serovar 4b appears to be responsible for most cases of listeriosis despite the fact that serovars 1/2a, 1/2b, and 1/2c are more common in foods. Additionally, serovar 4b is associated more often with listeriosis in cases involving pregnancy (McLauchlin 1990a). Serovars 1/2a, 1/2b, 1/2c, 3a, and 4b have been associated with cases of both human and animal listeriosis, but serovar 4a is only associated with disease in animals (Wiedmann *et al.* 1997).

The main route of entry for *L. monocytogenes* is through the mouth. For animals, this probably occurs frequently due to the widespread nature of the organism in the environment and in feed (Vázquez-Boland *et al.* 2001). For humans, a variety of raw foods have been implicated in listeriosis outbreaks, but cross-contaminated processed foods also pose a serious risk (McLauchlin 1996). The number of pathogenic bacteria that need to be ingested to cause listeriosis is unknown. In mice, the infectious dose is as low as 10^3 cells administered orally (Golnazarian *et al.* 1989), but it is as high as 10^9 cells in primates (Farber *et al.* 1991). The minimum infectious dose for humans is unknown, but outbreaks involve foods that are typically contaminated with 10^6 CFU/g of *L. monocytogenes* (Vázquez-Boland *et al.* 2001). Infectious dose can be influenced by factors such as host susceptibility and strain virulence, but Vázquez-Boland *et al.* (2001) point out that limitations in detection of *L. monocytogenes* can also play a role. In a case

of invasive listeriosis, as many as 30 days can pass between ingestion of the contaminated food and onset of illness (Linnan *et al.* 1988). The level of contamination of the food, as well as the infectious dose, might then be over-estimated (Vázquez-Boland *et al.* 2001).

After ingestion, *L. monocytogenes* must then pass through the stomach. It is thought that the acidity of the stomach is sufficient to kill a large number of bacteria; however, antacids and the buffering capacities of some foods may help the organism to survive (McLauchlin 1997). The organism also possesses a glutamate decarboxylase system that is essential for survival in gastric fluid (Cotter *et al.* 2001a).

It is in the small intestine where *L. monocytogenes* can exit the gastrointestinal system to spread to other organs. The translocation of *L. monocytogenes* across the intestinal epithelium can occur in either of two ways, although there is no agreement on which mechanism is used, if not both (Vázquez-Boland *et al.* 2001). One study has shown that the bacterium is capable of directly invading intestinal epithelial cells (Racz *et al.* 1972). However, studies in mice have shown that *L. monocytogenes*, like some other invasive pathogens, escapes from the intestinal lumen using the M cells in the Peyer's patches (Jensen *et al.* 1998). Regardless of how *L. monocytogenes* escapes the intestinal lumen, it is the next step that determines the type of illness (febrile gastroenteritis or invasive listeriosis) that will develop. In cases of enteritis, it is thought that once the bacteria gain access to the intestinal epithelium, they spread to neighbouring enterocytes but do not disseminate to other parts of the body (Vázquez-Boland *et al.* 2001). During invasive listeriosis, the bacteria cross the epithelium and enter the blood, where they are transported rapidly to other organs in the body such as the liver and spleen (Pron *et al.* 1998). For many years, it was thought that macrophages were the major host cell for *L. monocytogenes*, but now the hepatocytes of the liver are considered the main target tissue and site of multiplication (Rosen *et al.* 1989, Vázquez-Boland *et al.* 2001). If the host is pregnant, bacteria in the mother's blood can penetrate the placenta and infect the fetus (McLauchlin 1997). *L. monocytogenes* is also able to grow in amniotic fluid, and this can serve as a source of contamination in cases of late-onset neonatal listeriosis (McLauchlin 1997). The blood also serves to transport bacteria to the brain, spinal cord, and

meninges, where *L. monocytogenes* can establish foci of infection (Vázquez-Boland *et al.* 2001).

L. monocytogenes is an invasive pathogen, and its ability to enter, survive, and grow in cells is important to its ability to cause disease. Although human cells such as M cells and macrophages will normally phagocytose bacteria, *L. monocytogenes* must induce internalization to gain entry to non-professional phagocytes such as hepatocytes or enterocytes. The first step in invasion is attachment of the bacterium to the surface of the target cell. Unlike other intracellular pathogens, such as *Salmonella* and *Shigella* spp., *L. monocytogenes* does not induce membrane ruffling; instead, it appears to sink into the target cell until it is eventually engulfed (Mengaud *et al.* 1996). The exact mechanism for internalization is unknown, but the main bacterial mediators of the process have been identified. Internalins A and B are surface proteins of *L. monocytogenes* that are required for invasion of enterocytes (Gaillard *et al.* 1991) and hepatocytes (Dramsı *et al.* 1995) respectively. As the bacterium is engulfed by the cell, it is encased in a phagosomal vacuole that is also known as a primary phagosome (Vázquez-Boland *et al.* 2001).

L. monocytogenes uses a hemolysin and two enzymes with phospholipase C activity to escape the phagosome and enter the host cytoplasm (Jenkins *et al.* 1964, Camilli *et al.* 1993). The hemolysin listeriolysin O (LLO) is related to streptolysin O of *Streptococcus pyogenes* and is encoded by the *hly* gene (Jenkins *et al.* 1964). PlcA, which is encoded by the *plcA* gene, is specific for phosphatidylinositol; PlcB, however, is encoded by *plcB* and has a broader substrate specificity that includes phosphatidylcholine (Camilli *et al.* 1993). The hypothesized action of LLO in the primary phagosome is to form pores in the membrane which then allow the phospholipases to access their substrates (Vázquez-Boland *et al.* 2001). Interestingly, PlcA seems to have only a minor role in comparison to the requirement for PlcB in phagosomal escape. However, PlcA seems to have synergistic activity with PlcB and a metalloprotease (Mpl) of *L. monocytogenes* during escape from phagosomes (Marquis *et al.* 1995).

L. monocytogenes multiplies rapidly in the cytoplasm of the host cell. Actin filaments begin polymerizing on the surface of the bacterium, forming long tails at one pole as the bacteria divide and new cell wall is synthesized (Kocks *et al.* 1993). The bacterial surface protein ActA is responsible for recruiting actin to the cell surface, and

mutations in the *actA* gene can abolish intracellular motility and cell-to-cell spread (Kocks *et al.* 1992). The actin tail essentially pushes the bacterium around in the host cell (Tilney and Portnoy 1989). Eventually, the bacterium reaches the cell membrane, at which point it continues to push outwards, forming a pseudopod-like protrusion which penetrates into a neighbouring cell until it is phagocytosed (Tilney and Portnoy 1989). The result is the formation of a double-membrane secondary phagosome which is composed of an inner membrane from the original donor cell and an outer membrane from the new host cell (Tilney and Portnoy 1989). The virulence factors required for escape from the primary phagosome are the same as those needed for the secondary phagosome despite differences in lipid composition and the presence of an additional membrane (Vázquez-Boland *et al.* 2001). After escaping from the secondary phagosome, the bacterium can then multiply in the cytoplasm of the new host cell, accumulate actin tails, and resume the infectious cycle of cell-to-cell spread (Tilney and Portnoy 1989).

2.1.3 Case studies

Although sporadic cases of listeriosis had been reported for many years, the illness was not a major concern for food safety until several large outbreaks occurred in North American and Europe. The first of these outbreaks occurred in Boston in 1979 (Ho *et al.* 1986). During this outbreak, 20 adults contracted listeriosis, and three of them died. Contaminated raw vegetables, which were served with a variety of suspected foods, are suspected as the probable source of *L. monocytogenes*, although the investigators were unable to rule out pasteurized milk as a source. The strain belonged to serovar 4b.

In Canada, the first large outbreak of listeriosis occurred in the Maritime provinces in 1981 (Schlech *et al.* 1983). In this outbreak, there were 41 cases of listeriosis, but only seven of the patients were adults. The remaining 34 were perinatal cases of listeriosis, and nine of these resulted in stillbirth. Infants were born with listeriosis in 23 of the cases, and the remaining two infants were born healthy. The mortality rates for the adult and perinatal cases were similar (28.6% and 27%, respectively). *L. monocytogenes* serovar 4b was responsible for the outbreak, and the implicated food was coleslaw. The coleslaw had been prepared locally from cabbage that

was grown in fertilized fields. The fertilizer included manure from a flock of sheep that had previously contained animals infected with listeriosis.

In 1983, there was an outbreak of listeriosis in Massachusetts which involved 49 people (Fleming *et al.* 1985). Forty-two of the cases were adults, and 7 were perinatal. There were 14 deaths that were eventually linked to consumption of pasteurized milk. The milk was traced back to farms where cows had listeriosis, but there was no evidence that the milk had been improperly pasteurized. As a result, the investigators questioned the suitability of pasteurization for killing large numbers of *L. monocytogenes* due to potential protective properties of the milk.

One of the largest outbreaks of invasive listeriosis occurred in California in 1985 (Linnan *et al.* 1988). There were a total of 142 cases, 93 of which were perinatal, and the other 49 were adults. Of the 48 deaths that occurred, 30 were perinatal cases, and 18 were adults. The source of the outbreak was Mexican-style soft cheese contaminated with *L. monocytogenes* serovar 4b. Although the pasteurizing equipment was not faulty, phosphatase tests of the cheese indicated inadequate pasteurization.

Another large outbreak occurred in the United States in 1998 and spanned 24 states (Anonymous 1998, Graves *et al.* 2005). In this outbreak, 108 people contracted listeriosis and there were 18 deaths, four of which were stillbirths or miscarriages. Investigators determined that the source of bacteria was hot dogs that were contaminated in a processing plant. The outbreak strain was *L. monocytogenes* CDC 7762, which was used in this study.

Several outbreaks of febrile gastroenteritis involving *L. monocytogenes* have also been reported. One of the first outbreaks occurred in New York City in 1989 (Riedo *et al.* 1994). Ten adults attending a party, including two pregnant women, contracted febrile gastroenteritis. The source could not be identified, but the investigators suggested shrimp was the most likely candidate. The outbreak strain belonged to serovar 4b.

In 1997, a large outbreak of listeriosis in Northern Italy sickened 1566 school children and adults. Children reported fever and vomiting more frequently than adults, who experienced diarrhea and joint pain more often. Symptoms were so severe for 292 of the children, that they were admitted to hospital. The outbreak was associated with

canned corn that was likely contaminated with a serovar 4b strain during the preparation of a corn salad dish (Aureli *et al.* 2000).

More recently, an outbreak in Los Angeles occurred in 2001 (Frye *et al.* 2002). Sliced turkey from a deli was implicated in 16 cases of febrile gastroenteritis in adults attending a party. The strain in this outbreak belonged to serovar 1/2a.

In 2002, there was an outbreak of listeriosis involving turkey deli meat (Anonymous 2002). In eight states, there were a total of 46 cases, ten of which were fatal (three stillbirths/miscarriages, seven adults). The most likely source was cross-contamination the environment of the processing plant.

2.1.4 Control in foods

In the food industry, contamination of products with pathogens can have consequences for public health and for economic loss from product recalls and damage to reputation. *L. monocytogenes* is found naturally in some food products such as raw milk, but its ubiquitous presence in the environment also means there is potential for cross-contamination of foods that should not be associated with the bacterium (FDA 1991). Arguably the most important tool for control of *L. monocytogenes* in foods is a Hazard Analysis Critical Control Point (HACCP) plan for identification of potential problems and coordination of measures to manage the problem of contamination by *L. monocytogenes* and other pathogens (FDA 1991).

Depending on the food product, there are various techniques available for eliminating pathogens such as *L. monocytogenes*. After slaughter, carcasses can be chemically decontaminated with organic acids such as acetic, lactic, and citric acid (Dincer and Baysal 2004). Meat, both raw and processed, can also be decontaminated by steam pasteurization (Dincer and Baysal 2004). Fermented meat products contain enough lactic acid, salt, and nitrite to effectively inhibit the growth of *L. monocytogenes* (WHO/FAO 2004). Ionizing radiation is a new technology that is seeing limited use for decontamination of meats, but it is effective at killing *L. monocytogenes* and other pathogens (Dincer and Baysal 2004). It has the added advantage that the product can be

irradiated after packaging, so post-processing contamination can also be eliminated (Dincer and Baysal 2004).

In dairy products, *L. monocytogenes* can be eliminated by pasteurization (WHO/FAO 2004). The bacterium can persist in ripened, fermented, and cultured dairy products, and there is a health risk associated with consumption of dairy products made with raw milk (Farber and Peterkin 1991). Growth of *L. monocytogenes* in dairy products can be reduced - but not stopped - by low pH and competition from background microflora (Farber and Peterkin 1991).

Although there are interventions that can be used by the food industry to limit contamination and growth of *L. monocytogenes* in foods, these methods have not provided the food industry with assurance that the organism can be sufficiently controlled to eliminate cases of listeriosis. Alternative strategies using novel techniques, such as the use of bacteriocins, are needed to ensure the safety of the food supply.

2.2 Bacteriocins

Bacteriocins are peptides or proteins with bacteriocidal or bacteriostatic properties. They are transcribed to mRNA and ribosomally synthesized by bacteria to kill or inhibit the growth of other bacteria. One of the main features of bacteriocins that differentiates them from antibiotics is that most antibiotics are not proteinaceous, and those that are, are not synthesized by ribosomes (Cleveland *et al.* 2001). In addition, bacteriocins have a relatively narrow activity spectrum that is comprised of closely-related bacteria. Bacteriocins are produced by both Gram-positive and Gram-negative bacteria, although bacteriocins synthesized by the latter are termed colicins.

Interest in bacteriocins has risen in recent years. Driving this interest is an increase in consumer demand for minimal processing of foods, which has led to exploration of alternative preservation technologies for use in the food industry (Devlieghere *et al.* 2004). Biopreservation techniques include the addition of bacteriocins or bacteriocin-producing cultures to foods. The bacteriocins produced by lactic acid bacteria (LAB) are particularly suitable for biological preservation because

there are many producer strains that are non-pathogenic and occur naturally in foods. To date, the only bacteriocins approved for use in foods are nisin and pediocin.

The genes for bacteriocin production are usually organized in clusters and can be located on either the chromosome or on a plasmid (Cleveland *et al.* 2001). In the most basic form, the required genes are the structural gene of the bacteriocin, and an immunity gene (van Belkum and Stiles 2000). Since bacteriocins are active against closely-related microbes, the immunity gene is required to protect the producer cell from the antibacterial activity of its own bacteriocin. However, many bacteriocins require post-translational modification, regulatory proteins, or their own dedicated transporter, so additional genes are usually present in the operon.

2.2.1 Classification of bacteriocins

Classification of the bacteriocins produced by LAB is based on a scheme devised by Klaenhammer (1993) in which each class of bacteriocin is defined by characteristics such as size, amino acid composition, and heat stability. In total, Klaenhammer (1993) divided LAB bacteriocins into four main classes as follows:

Class I bacteriocins are the lantibiotics, so named because they all contain uncommon amino acids such as lanthionine and β -methyl lanthionine. They are small peptides <5 kDa in size. Examples of class I bacteriocins are nisin (Harris *et al.* 1992) and lactacin 481 (Piard *et al.* 1992)

Class II bacteriocins are <10 kDa and range from moderately to highly heat stable. The class is further divided into subclasses a, b, and c (Klaenhammer 1993). Class IIa bacteriocins are known as *Listeria*-active, since they inhibit or kill *L. monocytogenes*. They all contain an N-terminal amino acid sequence YGNGVXC. Leucocin A is an example (Hastings *et al.* 1991). Class IIb bacteriocins are two-component bacteriocins which require both peptides for full activity, such as lactacin F (Allison *et al.* 1994). The class IIc bacteriocins are peptides that contain cysteine residues that must be reduced to activate the bacteriocin. An example is lactococcin B (Venema *et al.* 1993).

Class III bacteriocins are >30 kDa and their only other characteristic is that they are heat labile. An example is lactacin A (Toba *et al.* 1991).

Class IV bacteriocins include peptides that contain lipids and/or carbohydrates in their structure, unlike the other classes which are composed entirely of amino acids. One such bacteriocin is plantaricin S (Jiménez-Díaz *et al.* 1993).

Based on the results of more recent work, other investigators have suggested modifications to Klaenhammer's (1993) original classification scheme, although much of it remains intact. Some more modern schemes subdivide the lantibiotics into types A and B based on structural differences among the class I bacteriocins (Cintas *et al.* 2001). Type A lantibiotics are elongated, non-globular peptides such as nisin. The type B lantibiotics, like mersacidin, are globular peptides. In addition, the class I bacteriocins are sometimes referred to as the "modified bacteriocins" since this is the only class containing peptides that are extensively modified after translation (Hécharad and Sahl 2002). Accordingly, the class II bacteriocins are considered "unmodified".

In their review on bacteriocins, Nes *et al.* (1996) propose that since research has shown that cysteine in lactococcin B can be replaced with some other amino acids without loss to activity, class IIc is no longer appropriate. Instead, the secretion of bacteriocins should be added to the criteria used for classification. Most class II bacteriocins are transported out of the cell via dedicated ATP-binding cassette (ABC) transporters, and the characteristic leader peptide contains two glycine residues. However, there are bacteriocins such as divergicin A which use the general secretory pathway, so according to Nes' classification scheme, class IIc should be re-defined to contain only these sec-dependent proteins (Nes *et al.* 1996). Still other researchers suggest that class IIc should be re-designated for all of the class II bacteriocins that simply do not fit well into classes IIa and IIb (Hécharad and Sahl 2002).

In addition, the bacteriocins that were classified by Klaenhammer (1993) as class IV, were not well studied and only partially purified at that time. For better characterization of those bacteriocins, further purification has been suggested (Nes *et al.* 1996, Cintas *et al.* 2001). Indeed, the class IV bacteriocin plantaricin S was purified and the antimicrobial activity is now attributed to two peptides rather than a glycolipid as

previously thought (Cintas *et al.* 2001). These findings raise the issue of the suitability of having a separate class for bacteriocins which may simply require more extensive study.

The classification scheme of van Belkum and Stiles (2000) is distinct from most others due to its focus on using cysteine residues and peptide secondary structure to subdivide class II. Class IIa is defined as bacteriocins containing four cysteine residues, resulting in two disulfide bridges (van Belkum and Stiles 2000). Classes IIb and IIc both contain two cysteines (one disulfide bridge), but the former has a disulfide bridge in the N-section of the peptide, whereas the bridge of the latter connects the N- and C-sections (van Belkum and Stiles 2000). Class IId consists of peptides with one or zero cysteine residues (no disulfide bridge); class IIe are the two-peptide bacteriocins, and class IIf are class II bacteriocins that do not fit into the other classes (van Belkum and Stiles 2000). This classification scheme is not encountered as commonly as the class II definitions of Klaenhammer (1993), Nes *et al.* (1996), and Héchard and Sahl (2002).

Since so many variations of Klaenhammer's classification scheme exist, the class definitions that will be used for the remainder of this review are summarized below in Table 1. Little is known about the action of class III bacteriocins, so they will not be discussed further.

Table 1: Classification of bacteriocins of lactic acid bacteria (modified from Cintas *et al.* 2001 and Héchard and Sahl 2002)

Class	Subclass	Characteristics	Representative Bacteriocins
I	A	Non-globular lantibiotics	nisin
	B	Globular lantibiotics	mersacidin
II	a	<10 kDa, <i>Listeria</i> -active YGNGVXC motif	leucocin A, pediocin
	b	<10 kDa, two-component bacteriocins	brochocin-C
	c	<10 kDa, Non IIa/IIb bacteriocins	plantaricin A

2.2.2 Mode of action

Although there are many different bacteriocins produced by Gram-positive bacteria, they are thought to share some general mechanisms for their antibacterial activity. The majority of these bacteriocins kill or inhibit bacterial growth by associating with multiple bacteriocin subunits to form pores in the membrane of the target cell. Depending on the bacteriocin, this may require the presence of specific receptors on the target cell, or it may occur in a receptor-independent process. The formation of pores in the membrane has a number of consequences, such as loss of proton motive force and dissipation of membrane potential. Efflux of amino acids, ATP, and ions can also occur, depending on the bacteriocin (Maftah *et al.* 1993, Chen and Montville 1995, Moll *et al.* 1999b).

Type A lantibiotics such as nisin are amphiphilic. When they come in contact with a membrane, their hydrophilic amino acids interact with the hydrophilic phospholipid heads, and the hydrophobic regions insert into the lipid bilayer (Driessen *et al.* 1995). No specific receptor is required for the bacteriocin, although inhibition is thought to occur only at high (μM) concentrations of bacteriocin (Hécharde and Sahl 2002). The insertion of multiple bacteriocin-molecules into a region of the membrane results in the formation of a transient pore (Moll *et al.* 1999a).

At lower (nM) concentrations, some type A lantibiotics like nisin have been shown to bind to lipid II (Breukink *et al.* 1999). Lipid II, a component of the cell membrane, is a peptidoglycan precursor that is involved in the synthesis of the bacterial cell wall. Nisin binds specifically to lipid II, using it as a docking molecule prior to insertion into the membrane (Hasper *et al.* 2004). There is recent evidence that the pore complexes formed by nisin include lipid II molecules as an integral, stabilizing component in their structure (Hasper *et al.* 2004). Furthermore, these bacteriocins exhibit an antimicrobial activity in addition to membrane permeabilization, since the binding of these bacteriocins to lipid II interferes with peptidoglycan synthesis (Linnett and Strominger 1973).

A third antimicrobial activity of type A lantibiotics involves enzymatic destruction of the staphylococcal cell wall and subsequent autolysis of the target cell. The cationic enzymes *N*-acetylmuramoyl-L-alanine amidase and an *N*-

acetylglucosaminidase are normally bound to the cell walls of susceptible strains of staphylococci. In this bound state, they are inactive; however, they can be displaced by the cationic bacteriocins nisin and Pep5, and subsequently become active. The result is rapid degradation of the cell wall and death of the cell (Bierbaum and Sahl 1987).

The type B lantibiotics also have a variety of antimicrobial activities. Mersacidin and actagardine are also capable of binding to lipid II and inhibiting cell wall synthesis. Unlike the type A lantibiotics, mersacidin and actagardine bind to a different site on lipid II, and do not form pores and permeabilize membranes (Brötz *et al.* 1998).

Cinnamycin is a type B lantibiotic that has the ability to form pores in target membranes. Not much is known about these pores, but it has been suggested that they may involve phosphatidylethanol-amine in the target cell membrane, since cinnamycin is capable of binding to this molecule (Märki *et al.* 1991).

The class II bacteriocins all have a similar mode of action that results in permeabilization of the membrane. These peptides form pores in the membranes of target cells, and they are active in nanomolar concentrations (Hécharde and Sahl 2002). Their activity at low concentrations suggests the presence of a cell surface receptor, much like the case of nisin binding to lipid II in nanomolar amounts.

Among the class IIa peptides, only one putative receptor has been suggested. Studies with *L. monocytogenes* has revealed that mutations to the *mptACD* operon were responsible for resistance to mesentericin Y105 (Dalet *et al.* 2001). The operon encodes for the second enzyme (EII_t^{Man}) of a mannose-specific phosphotransferase system (PTS), and it is thought that EII_t^{Man} may actually function as a cell surface receptor for mesentericin Y105 (Dalet *et al.* 2001, Gravesen *et al.* 2002b). Furthermore, studies with other class IIa bacteriocins have yielded results that suggest EII_t^{Man} could be a receptor for all peptides in this class. An *mptA* knockout mutant of *L. monocytogenes*, which was unable to express one of the EII_t^{Man} subunits, was resistant not only to mesentericin Y105, but also pediocin PA-1 and leucocin A (Gravesen *et al.* 2002b). Other changes in gene expression have also been related to the *mptACD* operon. Expression of the operon depends on the transcription factor σ^{54} , so its expression can be abolished by mutations in the σ^{54} gene *rpoN*, or its activator *manR* (Robichon *et al.* 1997, Dalet *et al.* 2001). In addition, it is thought that *mptACD* expression can regulate the expression of other PTS,

which may explain the upregulation of a β -glucoside-specific PTS and a phospho- β -glucosidase that was observed in pediocin-resistant *L. monocytogenes* (Gravesen *et al.* 2000).

The two-peptide bacteriocins in class IIb function by forming pores in target membranes, but they are only active when both parts of the heterodimer are present (Klaenhammer 1993, McCormick *et al.* 1998). The effects on the target cell are similar to those produced by other pore-forming bacteriocins: disruption of membrane potential, proton motive force, intracellular pH, and efflux of ions and amino acids (Héchar and Sahl 2002).

The class IIc bacteriocin plantaricin A disrupts the membrane potential and intracellular pH of target cells (Hauge *et al.* 1998). Lactococcin A also affects membrane permeability, but it dissipates membrane potential and affects movement of amino acids into and out of susceptible cells (van Belkum *et al.* 1991). Lactococcin 972 is a two-peptide bacteriocin, but it is composed of two identical peptides unlike the class IIb bacteriocins (Martínez *et al.* 1996). It does not affect membrane permeability, and instead acts by preventing the incorporation of *N*-acetylglucosamine into newly synthesized cell walls, thus inhibiting septum formation and binary fission (Martínez *et al.* 2000).

2.2.3 Bacteriocin immunity

Bacteriocins function against species of bacteria that are closely related to the producer strain, which requires that the producer possess some mechanism for resisting its own antimicrobial peptide. For the non-lantibiotics, a specific immunity gene is typically located near other bacteriocin-related genes (Nes *et al.* 1996), and encodes a peptide that confers immunity upon the producer. For the lantibiotics, however, there exists a more complex interaction between the immunity protein and other gene products (Cleveland *et al.* 2001).

The immunity proteins of the class II bacteriocins share very little sequence homology with each other, even in cases where the corresponding bacteriocins are very similar (Nes *et al.* 1996). For example, the bacteriocins sakacin A and curvacin A are

identical, but their immunity proteins differ in size by 39 amino acids (Nes *et al.* 1996). The lack of homology among immunity proteins has led some to conclude that there is no direct physical interaction between these immunity proteins and bacteriocins (Nes *et al.* 1996, Ennahar *et al.* 2000).

The mechanism of bacteriocin immunity is still unknown, but a recent study focusing on the pediocin-like bacteriocins enterocin A and leucocin A has demonstrated that the C-terminal end of their immunity proteins appear to be responsible for bacteriocin recognition (Johnsen *et al.* 2004). Furthermore, their findings suggest that the immunity proteins of class IIa bacteriocins do not integrate into the cell membrane, but may still be capable of associating with the membrane as peripheral membrane proteins (Johnsen *et al.* 2004).

In contrast to the class IIa bacteriocins, the immunity protein of the class IIc bacteriocin lactococcin A does have the ability to insert into the cell membrane. There is also speculation that the immunity protein interacts with a proposed lactococcin A receptor in the cell membrane, thus preventing pore formation (Venema *et al.* 1994).

Unlike class II bacteriocins, immunity to lantibiotics is not solely dependent on the presence of a single immunity gene. For nisin, the immunity gene contributes to immunity, but there are additional genes which also impart some degree of resistance, even in the absence of the immunity gene (Cleveland *et al.* 2001). The actual mechanism of immunity to class I bacteriocins is still unknown (Cleveland *et al.* 2001).

2.2.4 Bacteriocin resistance

Many species of bacteria are inherently resistant to certain bacteriocins; for example, the outer membrane renders Gram-negative organisms resistant to LAB bacteriocins. However, it is also possible for previously susceptible strains to acquire resistance. This characteristic is distinct from bacteriocin immunity, which arises from expression of an immunity protein by the bacteriocin-producing strain. In terms of using bacteriocins for biopreservation, there is little sense in developing bacteriocins for use in foods if susceptible populations can easily become resistant to them. Resistance to a bacteriocin is also closely linked to the mechanism that the bacteriocin uses to kill or

inhibit target microbes. It is therefore important to understand how and why resistance can develop.

In terms of resistance to bacteriocins, the general consensus is that in any given population of susceptible bacteria, there is a small subpopulation of cells that are naturally resistant to a particular bacteriocin (Ennahar *et al.* 2000). Upon exposure of the population to a bacteriocin, it is these bacteriocin-resistant cells which are able to survive the exposure to become the dominant phenotype (Ennahar *et al.* 2000).

Information on the nature of bacteriocin resistance is incomplete, although much research has been done with nisin. Since the site of action of bacteriocins is believed to be the cell surface, studies on bacteriocin resistance have focused on changes in the cell membrane. In theory, terminated expression of the nisin receptor, lipid II would result in resistance to receptor-mediated pore formation; however, this scenario is not possible given the importance of this molecule in cell wall synthesis and hence, cell viability (Gravesen *et al.* 2004). Investigators have found that the plasma membrane of nisin-resistant *L. monocytogenes* has altered fatty acid and phospholipid content, which serves to decrease membrane fluidity and presumably interferes with the insertion of nisin into the membrane of these organisms (Ennahar *et al.* 2000). In nisin resistant *L. monocytogenes*, there also appear to be changes in the cell wall that result in decreased sensitivity to lysozyme and increased sensitivity to some antibiotics, but the details have yet to be elucidated (Gravesen *et al.* 2004). A penicillin-binding protein has also been implicated in nisin resistance, possibly by blocking access to lipid II (Gravesen *et al.* 2004).

In recent years, the focus has shifted from nisin resistance toward the study of bacteria that are resistant to the class IIa bacteriocins. For *L. monocytogenes*, resistant strains have been grouped into two main categories (Vadyvaloo *et al.* 2002). The intermediately-resistant strains have a 50% inhibitory concentration (IC_{50}) 2.4 to 4 times higher than that of the sensitive strains. The IC_{50} of highly-resistant strains is more than 500 times that of sensitive strains (Vadyvaloo *et al.* 2002). The distinction between resistance levels is necessary because different resistance mechanisms are thought to be involved at each level.

The fluidity of the cell membrane of *L. monocytogenes* plays a role in resistance to class IIa bacteriocins (Vadyvaloo *et al.* 2002). Unlike the cell membranes of nisin-resistant *L. monocytogenes*, cells that are resistant to leucocin A do not have a more rigid membrane than sensitive cells (Ennahar *et al.* 2000, Vadyvaloo *et al.* 2002). In particular, the phospholipids in the membranes of resistant cells had shorter, unsaturated acyl chains that increase membrane fluidity (Vadyvaloo *et al.* 2002). The increased fluidity is thought to contribute to resistance to leucocin A by interfering with insertion of bacteriocin molecules into the cell membrane, and preventing formation of stable pores (Vadyvaloo *et al.* 2002). Increased membrane fluidity is thought to be involved in both intermediate and high levels of resistance to class IIa bacteriocins (Vadyvaloo *et al.* 2004a).

A recent study on cell surface charge of highly-resistant *L. monocytogenes* has shown that the teichoic acid has increased D-alanine content, which decreases the negative charge of the cell wall (Vadyvaloo *et al.* 2004a). Additionally, the increased presence of L-lysine was found in the phospholipids of highly-resistant cells (Vadyvaloo *et al.* 2004a). Specifically, cardiolipin and phosphatidylglycerol, which normally bear a negative charge, become positively charged when modified with a lysyl residue (Vadyvaloo *et al.* 2004a). The overall effect is that cells of *L. monocytogenes* that are highly-resistant to class IIa bacteriocins have a surface that is less negatively charged than that of sensitive or intermediately-resistant cells (Vadyvaloo *et al.* 2004a).

Perhaps the most important mediator of resistance to class IIa bacteriocins is gene expression from the *mptACD* operon (Gravesen *et al.* 2002b, Ramnath *et al.* 2004, Vadyvaloo *et al.* 2004a). As mentioned previously, these genes encode the subunits for the permease component (EII_t^{Man}) of a mannose-dependent PTS (Dalet *et al.* 2001, Héchard *et al.* 2001). For PTS permeases, there are three or four subunits; two are peripheral membrane proteins (IIA and IIB), and there are one or two (IIC and IID) integral transmembrane proteins (Kotrba *et al.* 2001). The *mptA* gene encodes subunit $EIIAB_t^{Man}$ which, like the mannose permease of *Escherichia coli*, is a fusion of the IIA_t^{Man} and IIB_t^{Man} subunits (Dalet *et al.* 2001, Héchard *et al.* 2001, Kotrba *et al.* 2001). The *mptC* and *mptD* genes encode the $EIIC_t^{Man}$ and $EIID_t^{Man}$ subunits, respectively (Héchard *et al.* 2001).

Studies to elucidate the role of each subunit in bacteriocin resistance have yielded conflicting results. Vadyvaloo *et al.* (2004a) used real-time PCR to show correlation between gene expression and the level of bacteriocin resistance. Highly-resistant strains of *L. monocytogenes* have large (1 000- and 3.6 million-fold) decreases in *mptA* expression compared with sensitive cells, and intermediately-resistant strains have a more moderate (four-fold) decrease (Vadyvaloo *et al.* 2004a). These results suggest that the level of *mptA* expression may be the primary factor that dictates the level of resistance to class IIa bacteriocins (Vadyvaloo *et al.* 2004a). In a different approach, Ramnath *et al.* (2004) investigated the *mptACD* operon using heterologous expression of *mpt* genes to induce bacteriocin sensitivity in the insensitive bacterium *Lactococcus lactis*. After expressing different combinations of the three genes in *L. lactis*, the authors found that *mptC* in any combination with the other *mpt* genes was capable of inducing sensitivity to class IIa bacteriocins, and it could even do so on its own (Ramnath *et al.* 2004). It might be beneficial if *mptC* expression were analyzed in the same manner as Vadyvaloo *et al.* investigated *mptA* (2004a) to determine if there is a similar correlation between *mptC* and resistance level. Since EIIC₁^{Man} is a transmembrane protein, it is conceivable that this subunit interacts with class IIa bacteriocins (Ramnath *et al.* 2004). The role of EIIB₁^{Man} is less clear, because this subunit is located peripherally on the cytoplasmic side of the cell membrane (Kotrba *et al.* 2001). Although EIIB₁^{Man} cannot interact directly with an extracellular bacteriocin, in *Streptococcus mutans* EIIB₁^{Man} deficiency can affect the expression of a variety of genes (Abranches *et al.* 2003).

In Gram-positive organisms, cross-resistance among some class IIa bacteriocins has been observed (Dalet *et al.* 2000), which is unsurprising considering the common receptor-mediated binding mechanism that they are thought to use (Gravesen *et al.* 2002b). Cross-resistance involving bacteriocins of different classes has also been documented, but the basis is not well understood (Crandall and Montville 1998, Gravesen *et al.* 2004). For example, a study by Crandall and Montville (1998) found that *L. monocytogenes* ATCC 700302, which is resistant to the class I bacteriocin nisin, was also resistant to the class IIa bacteriocin pediocin PA-1 and leuconocin S, which is classified as class IIc (see Table 1). The authors were unable to speculate on possible mechanisms for cross-resistance.

Gravesen *et al.* (2004) showed nisin resistance in strains of *L. monocytogenes* that were resistant to class IIa bacteriocins, but this was only present in three of eight highly-resistant mutants and one of four intermediately-resistant strains. Furthermore, none of the twelve strains exhibited the nisin resistance mechanism being studied – increased penicillin-binding protein expression (Gravesen *et al.* 2004). They also examined some nisin-resistant mutants displaying resistance to class IIa bacteriocins, and found that the cross-resistance in one mutant might be due to decreased *mptA* and *mptD* expression as well as increased D-alanine content in the cell wall teichoic acids (Gravesen *et al.* 2004). There is still much research that needs to be done to better understand cross-resistance involving nisin and class IIa bacteriocins.

Fitness costs are consequences of bacteriocin resistance that have some detrimental effect on the cell (Dykes and Hastings 1998). One such cost is reduced growth rate, which has been reported for strains of *L. monocytogenes* that are resistant to class IIa bacteriocins (Dykes and Hastings 1998, Vadyvaloo *et al.* 2004b). However, the cell population in stationary phase (i.e. the total biomass) was actually greater for the bacteriocin-resistant strains (Vadyvaloo *et al.* 2004b). The decrease in growth rate was linked with a 50% decrease in glucose consumption rate (Vadyvaloo *et al.* 2004b). These slow growing mutants did not express MptA (EIIAB_t^{Man}), which is typical in strains with a high level of bacteriocin resistance, and it was suggested that EII_t^{Man} might be the primary transporter for glucose in *L. monocytogenes* (Vadyvaloo *et al.* 2004b). Furthermore, the increased biomass of the resistant mutants was due to a switch to mixed acid fermentation of glucose, instead of the homolactic fermentation used by the wild type strains (Vadyvaloo *et al.* 2004b). Compared with homolactic fermentation, mixed acid fermentation produces one extra mol of ATP for every glucose, and the extra energy is manifested as increased biomass (Vadyvaloo *et al.* 2004b). In nisin- and pediocin-resistant *L. monocytogenes*, susceptibility to environmental stresses were investigated as possible fitness costs (Gravesen *et al.* 2002a). Decreased growth rate was also observed for the strains studied, although it was more pronounced with pediocin resistance (Gravesen *et al.* 2002a). Acquisition of pediocin resistance was unaffected by low temperature (10°C), lowered pH (5.5), or NaCl (6.5%); in contrast, development of nisin resistance was reduced by salt and low temperature stress, which is likely due to

differences in the mechanism and acquisition of resistance to class I and class IIa bacteriocins (Gravesen *et al.* 2002a). In a study where resistant cells were subjected to environmental stresses, nisin-resistant *L. monocytogenes* were found to be more sensitive to heat than the wild type (Modi *et al.* 2000).

2.3 Environmental stress

In any environment, bacteria can face harmful external stresses such as extremes of temperature and pH, low water activity (A_w), redox potential (E_H), and various antimicrobial compounds (Leistner 2000). Only those bacteria that are able to cope with environmental pressures will persist. In the environment of a food product, processors can manipulate these factors to control spoilage organisms and pathogens. Unfortunately, the amount of stress required to kill an organism (high salt content, for example), can sometimes change the sensory quality of the food to a degree that it is unacceptable to consumers (Leistner 2000). As an alternative, the hurdle concept involves combining several environmental stresses at sublethal levels to achieve the same level of control over undesirable microbes as a single lethal treatment, while maintaining the quality of the food (Leistner 2000).

The phenomenon of stress tolerance has implications for the hurdle concept. When bacteria are exposed to sublethal doses of an environmental stress, they can adapt and develop tolerance that allows them to survive a later challenge with higher doses of the same stress, and sometimes other stresses (Lou and Yousef 1997, Leistner 2000). Adaptation of *L. monocytogenes* to sublethal pH and ethanol concentrations increases tolerance to lethal levels of acid, ethanol, and H_2O_2 (Lou and Yousef 1997). H_2O_2 , NaCl, and heat stress can increase H_2O_2 tolerance, and heat shock can also increase tolerance to ethanol and NaCl (Lou and Yousef 1997). Cross-tolerance has also been demonstrated using *L. monocytogenes* that have been adapted to acid, resulting in a decreased heat tolerance in media containing glucose (Bayles 2003). *L. monocytogenes* is a prime example of a bacterium that possesses many mechanisms to tolerate a variety of environmental stresses (Lou and Yousef 1997, Hill *et al.* 2002).

2.3.1 General stress tolerance mechanisms

Although specialized tolerance mechanisms exist for different types of stress, many of them are controlled by more generalized responses. For a bacterium to respond to a stress, it first must be able to detect the change in its environment. Two-component regulatory systems are used by bacteria to sense and respond to external changes (Cotter *et al.* 1999). Two-component signal transduction systems consist of a sensory histidine kinase, which is associated with the cell membrane, to detect certain changes in the local environment, and a response regulator that modulates gene expression accordingly (Cotter *et al.* 1999). The first two-component system that was discovered to respond to stress in *L. monocytogenes* is *lisRK* (Cotter *et al.* 1999). A mutant with a deletion in the *lisK* gene exhibited a growth-phase dependent change in acid tolerance, becoming more sensitive in early log phase, but more resistant in stationary phase (Cotter *et al.* 1999). The mutant was also able to tolerate higher concentrations of ethanol, but its virulence was highly attenuated, and the authors suggested this might be due to a loss of control of virulence gene expression by *lisRK* (Cotter *et al.* 1999).

Alternate sigma factors allow for transcription of certain subsets of genes, and expression of some of these sigma factors is dependent on stress conditions (Ferreira *et al.* 2001). In Gram-negative and Gram-positive bacteria, σ^S and σ^B , respectively, are the alternate sigma factors that regulate gene expression during periods of environmental stress and at the start of the stationary phase of growth (Ferreira *et al.* 2001). In *L. monocytogenes*, σ^B -deficient mutants have increased sensitivity to low pH, H_2O_2 , and glucose depletion, which indicates an important role for σ^B in acid tolerance, resisting oxidative stress, and coping with carbon starvation (Ferreira *et al.* 2001). σ^B has also been implicated in mediating osmotolerance in the presence of high salt (Becker *et al.* 1998). The involvement of σ^B in heat tolerance is less clear. In *Bacillus cereus*, σ^B plays a role in the heat shock response (van Schaik *et al.* 2004), but early research with *L. monocytogenes* did not find a decrease in heat tolerance in the absence of σ^B expression (Ferreira *et al.* 2001). A more recent study, however, showed that a strain deficient in σ^B expression was more heat sensitive, so there may be additional strain-specific factors at work (Moorhead and Dykes 2003).

The growth phase of a culture is an important consideration in examining general stress responses, since stationary phase cells have been shown to be more resistant to a variety of unfavourable conditions including acid, heat, and oxidative stress (Davis *et al.* 1996, O'Driscoll *et al.* 1996, McMahon *et al.* 2000, Weeks *et al.* 2004).

2.3.2 Heat tolerance

The heat stress response of *L. monocytogenes* involves transcription of various heat shock proteins that function as molecular chaperones, protecting proteins from denaturation and ensuring proper folding at elevated temperatures (Hill *et al.* 2002). ATP-dependent proteases also play an important role during heat stress, because they are required for degradation of misfolded proteins (Nair *et al.* 2000). Much of the understanding of the heat tolerance of *L. monocytogenes* is based on the *B. subtilis* model that divides heat shock gene expression into several classes (Nair *et al.* 2000). In particular, two classes of heat shock genes have been studied in *L. monocytogenes*. The class I genes are the molecular chaperones *groES*, *groEL*, and *dnaK*, which are controlled by the HrcA repressor protein (Nair *et al.* 2000). The class III heat shock genes encode the energy-dependent protease subunits ClpC, ClpE, and ClpP under control of the repressor CtsR (Nair *et al.* 2000).

Expression of the DnaK and GroEL proteins has been observed after sub-lethal heat shock (Hill *et al.* 2002). In addition, Gahan *et al.* (2001) demonstrated that the *groESL* operon can be induced by low pH, bile salts, and ethanol, although its primary function is still in heat tolerance.

The role of ClpC in heat tolerance has been demonstrated by inactivation mutants (Rouquette *et al.* 1996). Growth of the mutants was impaired at 42°C, but also in iron-limiting conditions and in the presence of 2% NaCl (Rouquette *et al.* 1996). ClpE is also required for sustained growth at 42°C, but its expression is not influenced by environmental stresses (Nair *et al.* 1999). Instead, it is upregulated in the absence of ClpC, possibly to compensate for ClpC deficiency (Nair *et al.* 1999). Furthermore, mutants with deficiency in CtsR, the repressor of *clp* expression, display increased growth in the presence of NaCl and at 42°C (Nair *et al.* 2000).

A recently discovered gene, *htrA*, also plays a role in the heat stress response (Wonderling *et al.* 2004). Deletion mutants have reduced growth rates at elevated temperatures and are more sensitive to heat shock (Wonderling *et al.* 2004). HtrA has been identified as a stress protein in other genera of bacteria, and it is a serine protease that may play a similar role as the Clp-ATPases, assisting in the degradation of misfolded proteins (Wonderling *et al.* 2004). HtrA deficiency also results in increased sensitivity to NaCl and H₂O₂ (Wonderling *et al.* 2004).

2.3.2 Acid tolerance

During the infection process, passage through the acidic stomach of potential hosts is one of the environmental stresses that *L. monocytogenes* must be able to survive (Cotter *et al.* 2001a). Thus an acid tolerance response (ATR) is extremely important for this organism to succeed as a pathogen. Tolerance to low pH also allows this organism to persist in acidic foods (Gahan *et al.* 1996). Two key mechanisms for the ATR of *L. monocytogenes* have been described: proton export by the F₀F₁-ATPase, and proton transfer via the glutamate decarboxylase (GAD) system (Hill *et al.* 2002).

The F₀F₁-ATPase has two functions: in organisms with a complete electron transport chain, it can synthesize ATP in the presence of oxygen; and under anaerobic conditions, it can export protons out of the cell to create proton motive force (Cotter *et al.* 2000). The result of transporting protons out of the cell is an increase in the cytoplasmic pH, which can maintain homeostasis in acidic environments (Cotter *et al.* 2000). To study the role of the F₀F₁-ATPase in acid tolerance of *L. monocytogenes*, Cotter *et al.* (2000) inhibited its activity with *N, N'*-dicyclohexylcarbodiimide (DCCD). Treatment of acid-adapted cells with DCCD increased their sensitivity upon further exposure to acid, although they were still more tolerant than untreated, unadapted cells and treated, unadapted cells (Cotter *et al.* 2000). Although these results showed that the F₀F₁-ATPase is important in the ATR, the finding that treated, adapted cells were still more acid-tolerant than untreated, unadapted cells suggested the presence of another acid tolerance mechanism independent of the F₀F₁-ATPase (Cotter *et al.* 2000).

The glutamate decarboxylase system is the other mechanism that plays a major role in the ATR of *L. monocytogenes*. In this system, glutamate is imported into the cell via a specific transporter. In the cytoplasm, the decarboxylation reaction involves consumption of a free proton and its enzymatic transfer to glutamate to yield γ -aminobutyrate (GABA). A dedicated glutamate/GABA antiporter then exports the GABA from the cell, and the result is an increase in the intracellular pH (Cotter *et al.* 2001a). Cotter *et al.* (2001a) identified the genes *gadA*, *gadB*, and *gadC* in *L. monocytogenes*, and their function was also determined from homology with other organisms with GAD systems. The *gadA* and *gadB* genes encode two glutamate decarboxylases, while the glutamate/GABA antiporter gene is *gadC* (Cotter *et al.* 2001a). Mutations in each of the *gad* genes resulted in varying degrees of sensitivity to porcine and synthetic human gastric fluid, with as much as a six log reduction in survival of the *gadAB* deletion mutant compared with the wild type (Cotter *et al.* 2001a). The GAD system is also important for the survival of *L. monocytogenes* in acidic foods, even those with low glutamate levels (Cotter *et al.* 2001b).

2.3.3 Salt tolerance

Much of the research on osmotolerance of *L. monocytogenes* has focused on the transport of organic osmolytes, otherwise known as compatible solutes (Hill *et al.* 2002). Compatible solutes are small molecules that are highly soluble and do not interact with proteins in the cell (Hill *et al.* 2002). Accumulation of these molecules in the cytoplasm during high salt conditions helps to maintain osmotic balance in the cell, and they can also protect enzyme function in high temperature, freeze-thaw, and drying (Hill *et al.* 2002, Cetin *et al.* 2004). The three most important compatible solutes in *L. monocytogenes* are glycine betaine, carnitine, and proline (Hill *et al.* 2002). The Gbu and BetL transporters import glycine betaine and the OpuC transporter is specific for carnitine (Cetin *et al.* 2004). The transporters are encoded by the *gbuABC*, *betL*, and *opuC* genes respectively, all of which contain σ^B -dependent promoters that can be induced by osmotic stress (Cetin *et al.* 2004). Sleator *et al.* (2003) showed that mutants with deficiencies in the three transporters have reduced survival in high-salt foods, and

that certain osmolytes are more important depending on the food type and its carnitine and glycine betaine content. Proline accumulation in *L. monocytogenes* is controlled by its synthesis in the cell, since the bacterium does not seem to possess a high-affinity transporter like *B. subtilis* does (Hill *et al.* 2002). The importance of proline in osmotolerance is less certain than that of carnitine and glycine betaine because a *L. monocytogenes* mutant that overproduced proline was not more osmotolerant than its parent strain (Sleator *et al.* 2001).

3 Materials and Methods

3.1 Bacterial cultures

The bacterial strains used in this study are listed in Table 2. Unless otherwise stated, cultures were maintained under the following conditions. All cultures were stored at -80°C in growth medium containing 20% glycerol, and subcultures were made from the frozen stock and grown in broth medium under the appropriate conditions. After incubation and growth, another subculture was prepared and incubated prior to use in an experiment. *Listeria* spp. were grown in tryptic soy broth supplemented with 0.6% yeast extract [TSBYE; Becton, Dickinson and Company (BD), Franklin Lakes, NJ] for 24 h at 30°C. *Leuconostoc gelidum* UAL187 and *Carnobacterium maltaromaticum* UAL9 were grown overnight (16 h) in All Purpose Tween (APT, BD) broth at 25°C. *Escherichia coli* DH5α was grown with aeration in Luria-Bertani (LB, BD) broth for 16 h at 37°C. *E. coli* TOP10 cells were grown with aeration in LB broth containing 50 µg/mL kanamycin (Km; Sigma-Aldrich, St. Louis, MO) for 16 h at 37°C.

3.2 Bacteriocin purification

To obtain partially purified leucocin A, the method of Hastings (1991) was used, with some modifications. *L. gelidum* UAL187 was subcultured into APT broth with a 1% inoculum and incubated at 25°C for 24 h. An Erlenmeyer flask containing 500 mL APT broth was inoculated with 2% *L. gelidum* UAL187 and grown overnight at 25°C. The culture was centrifuged at 6 000 x g for 30 min at 4°C (Sorvall RC-5B; Beckman Coulter, Fullerton, CA) and the supernatant was decanted. The supernatant was heated at 68°C in a waterbath for 30 min to kill any remaining bacterial cells, and then loaded onto a 2.5 x 48.5 cm Supelite DAX-8 column (Sigma-Aldrich) for separation by chromatography. The column was washed with 1 L of 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich), followed by a stepped gradient of 1 L solutions of 20%, 40%, 60%, and

80% ethanol (Sigma-Aldrich) in 0.1% TFA. The fractions were collected, stored at 4°C, and tested for inhibitory activity using a spot-on-lawn assay (Ahn and Stiles 1990).

Table 2: Bacterial strains used in this study

Organism	Reference
<i>Listeria monocytogenes</i> ATCC 15313	ATCC ^a
<i>L. monocytogenes</i> ATCC 15313L1 ^e	This study
<i>L. monocytogenes</i> ATCC 15313L2 ^e	This study
<i>L. monocytogenes</i> ATCC 15313L3 ^e	This study
<i>L. monocytogenes</i> CDC 7762	CDC ^b
<i>L. monocytogenes</i> CDC 7762L1 ^e	This study
<i>L. monocytogenes</i> CDC 7762L2 ^e	This study
<i>L. monocytogenes</i> CDC 7762L3 ^e	This study
<i>L. monocytogenes</i> FS 15	UAFMCC ^c
<i>L. monocytogenes</i> FS 15L1 ^e	This study
<i>L. monocytogenes</i> FS 15L2 ^e	This study
<i>L. monocytogenes</i> FS 15L3 ^e	This study
<i>Listeria innocua</i> ATCC 33090	ATCC ^a
<i>Leuconostoc gelidum</i> UAL187	(Hastings <i>et al.</i> 1991)
<i>Carnobacterium maltaromaticum</i> UAL9	UALABCC ^d
<i>Escherichia coli</i> DH5α	Gibco BRL, Gaithersburg, MD
<i>E. coli</i> TOP10	Invitrogen Canada
<i>E. coli</i> JM1 ^f	This study
<i>E. coli</i> JM2 ^f	This study
<i>E. coli</i> JM3 ^f	This study
<i>E. coli</i> JM4 ^f	This study

^aATCC = American Type Culture Collection

^bCDC = Centers for Disease Control and Prevention

^cUAFMCC = University of Alberta Food Microbiology Culture Collection

^dUALABCC = University of Alberta Lactic Acid Bacteria Culture Collection

^eLeucocin-resistant variant created from corresponding wild type strain

^f*E. coli* TOP10 containing plasmid pJM1, pJM2, pJM3, or pJM4

For the spot-on-lawn assay, two-fold serial dilutions were prepared using sterile MilliQ water as diluent. A total of 12 dilutions were made for each fraction. Ten μL of each dilution was spotted onto APT agar and allowed to air dry. Pronase E [5 μL of 1 mg/mL (Sigma-Aldrich)] was spotted adjacent to each dilution to verify the proteinaceous nature of any inhibitory compounds. After incubation for 30 min at 25°C, the plate was overlaid with 7 mL soft APT agar (0.75% agar, BD) that was inoculated with 1% *C. maltaromaticum* UAL9. Plates were incubated at 25°C for 16 h. The activity of each fraction, measured in arbitrary units (AU) was calculated as the reciprocal of the highest dilution showing a clear zone of inhibition of the indicator strain.

The most active fraction was concentrated to ~50 mL with a Büchi Rotavapor R-205 rotary evaporator (Büchi Labortechnik, Flawil, Switzerland). Trifluoroacetic acid (1%) was added to a final concentration of 0.1%. The partially purified bacteriocin was filtered with a low protein binding filter (Millex GV 0.22 μm , Millipore, Billerica, MA), and the resulting partially purified leucocin A was stored in 1 mL aliquots at -80°C.

3.3 Isolation of leucocin-resistant *Listeria monocytogenes*

To create leu^R *L. monocytogenes*, strains ATCC 15313, CDC 7762, and FS 15 were grown for 24 h at 30°C in TSBYE. After one subculture, the *L. monocytogenes* strains were inoculated (1%) into TSBYE containing 500 AU/mL leucocin A and grown at 30°C for 24 h. Each culture was plated onto tryptic soy agar supplemented with 0.6% yeast extract (TSAYE) containing 200 AU/mL leucocin A and incubated overnight at 30°C for 24 h. A total of six colonies were selected from each leucocin-supplemented agar plate and each was grown in unsupplemented TSBYE for 24 h, followed by another subculture and an additional 24 h incubation. The resistance of each culture was determined by a deferred inhibition assay (Tagg *et al.* 1976).

In the deferred inhibition assay, 1 μL of the leucocin A producer *L. gelidum* UAL187 was spotted onto APT agar and allowed to air dry. The plates were incubated for 16 h at 25°C and bacteria were killed with exposure to chloroform for 3 min.

Pronase E (5 μ L of 1 mg/mL) was spotted next to the colony and the plate was incubated for 30 min at 25°C. The plate was overlaid with 7 mL soft APT agar (0.75% agar) that was inoculated with 1% of a culture that had been isolated from agar plates supplemented with leucocin A. Plates were incubated for 24 h at 30°C, and the diameter of the zones of inhibition were measured. Cultures were considered to be resistant to leucocin if no inhibition zone was observed, or small zones (< 1 cm in diameter) were present that were unaffected by pronase E. Inhibition could be due to acid produced by *L. gelidum* UAL187.

A total of three leu^R variants were chosen for each strain of *L. monocytogenes*. Cultures were stored at -80°C in TSBYE and 20% glycerol. Each variant was given a designation from L1 to L3. To verify the stability of leucocin resistance, leu^R variants were subcultured and grown for 24 h in TSBYE in the absence of leucocin A. The process was continued each day for a total of four days, and deferred inhibition assays were performed on the fourth day to determine susceptibility of the cultures to leucocin A. The resistance of the wild type strains and leu^R variants was also tested with a spot-on-lawn assay (see section 3.2).

3.4 RAPD analysis of leucocin-resistant *Listeria monocytogenes*

To verify that the leu^R variants of *L. monocytogenes* were descended from the wild type parent strains, RAPD (randomly amplified polymorphic DNA) analysis was performed. DNA was extracted from bacteria using a modified protocol (Olson 2003) for the Wizard[®] genomic DNA purification kit (Promega Corporation, Madison, WI). One mL of a 24 h culture was aliquoted into a 1.5 mL microcentrifuge tube (Axygen, Union City, CA), centrifuged at 25°C (15 000 x g, 5 min; Eppendorf 5417 C/R centrifuge; Eppendorf AG, Hamburg, Germany), and the supernatant was discarded. The pellet was then resuspended in 480 μ L of 50 mM EDTA (ethylenediaminetetraacetic acid; Fisher Scientific, Edmonton, AB) and incubated with 120 μ L of 5 mg/mL lysozyme (Sigma-Aldrich) solution for 60 min at 37°C to degrade the bacterial cell wall. The sample was centrifuged at 25°C (15 000 x g, 3 min), and the supernatant was discarded. The pellet

was resuspended in 600 μ L of lysis solution (10 mM Tris-HCl, pH = 7.5; 10 mM EDTA, pH = 8; 0.5% SDS) and incubated at 80°C for 10 min. To degrade RNA, 6 μ L of RNase (2 mg/mL; Sigma-Aldrich) was added, and the sample was incubated at 37°C for 60 min. For protein removal, 200 μ L of 5 M ammonium acetate was added and mixed in by vortexing. After incubation on ice for 5 min, the sample was centrifuged at 25°C (15 000 x g, 3 min) and the supernatant was transferred to a sterile 1.5 mL microcentrifuge tube. DNA precipitation was performed by adding 600 μ L of isopropanol (VWR, Dorset, England). Tubes were inverted until the DNA strands became visible. After centrifugation at 25°C (15 000 x g, 3 min), the isopropanol was aspirated and the DNA was washed with 70% ethanol and centrifuged at 25°C (15 000 x g, 3 min). The ethanol was aspirated and the pellet was dried at 25°C. The DNA was dissolved in 100 μ L of TE buffer (10 mM Tris-HCl, pH = 7.5; 1 mM EDTA). Dissolution was aided by incubation at 65°C for 1 h, and tapping to mix every 20 min. The purified DNA was stored at -20°C until needed.

The primers M13 [5'-GAG GGT GGC GGT TCT-3'; (Hof *et al.* 2000)] and OPM-01 [5'-GTT GGT GGC T-3'; (Lawrence *et al.* 1993)] were used for RAPD analysis of each wild type strain and their leu^R variants. *Listeria innocua* ATCC 33090 was used as a control. For primer M13, reaction mixtures contained 1x PCR buffer, 4 mM MgCl₂, 100 μ M of each dNTP, 0.08 μ M primer M13, 2.5 μ L template DNA, and 1.25 U Taq polymerase (Invitrogen Canada, Burlington, ON). Reactions were amplified in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA) using a 2 min hot start at 94°C followed by 40 cycles of 94°C for 1 min, 45°C for 20 sec, and 72°C for 2 min, with a final hold at 72°C for 5 min. For primer OPM-01, reaction mixtures contained 1x PCR buffer, 1.5 mM MgCl₂, 200 μ M of each dNTP, 0.2 μ M primer OPM-01, 2.5 μ L template DNA, and 2.5 U Taq polymerase. After a 2 min hot start at 94°C, reactions were run for 44 cycles of 94°C for 1 min, 30°C for 2 min, and 72°C for 2 min, with a ramp time of 1 min between the annealing and extension phases. A final cycle of amplification consisted of 94°C for 1 min, 30°C for 2 min, and 72°C for 10 min.

For visualization, the PCR product was loaded onto a 2% agarose gel (Invitrogen Canada) containing 0.5 mg/mL ethidium bromide (EtBr; Bio-Rad, Hercules, CA). Gels were run at 5 V/cm for approximately 1.5 h and viewed under UV light with an Alpha DigiDoc™ digital documentation system (Alpha Innotech, San Leandro, CA). The molecular weight markers used were a 100 base-pair ladder (Invitrogen Canada) and lambda DNA digested with *Hind* III (0.5 µg/µL, Invitrogen Canada) and *Eco* RI (10 U/µL, Invitrogen Canada). Digestion with *Eco* RI was performed for 90 min at 37°C in a mixture containing 35 µg λ DNA/*Hind* III fragments, 50 U *Eco* RI, and 10% REact® 3 buffer (Invitrogen Canada).

3.5 Stress tolerance of leucocin-resistant *Listeria monocytogenes*

Wild type and leu^R variants of *L. monocytogenes* ATCC 15313, CDC 7762, and FS 15 were subcultured to TSBYE, incubated at 30°C for 24 h, and subcultured and incubated for an additional 24 h. To determine tolerance to environmental stress, the bacteria were subjected to conditions of high temperature, low pH, or high salt concentration. The number of survivors at each time point were plated in duplicate and data were converted to log CFU/mL and plotted against time. To determine differences in the mean D-values of the strains, each experiment was replicated three times, and D-values were calculated for each replicate. D-values were determined from the linear portion of the curve and represent the time required for a one log decrease in population. Statistical analysis of the D-values was performed using the Proc GLM procedure of SAS (SAS, Cary, NC) and means were separated with Duncan's multiple range test.

Prior to the first replicate of each experiment, the leucocin resistance of the leu^R variants and the sensitivity of the wild type strains was confirmed with a deferred inhibition assay using the procedure in section 3.3.

3.5.1 Heat tolerance

To determine the tolerance of *L. monocytogenes* to heat, a modified procedure of Aguilar (2004) was used. Cultures were exposed to sublethal heat to induce a heat shock response. One mL of a 24 h subculture of each strain was separately inoculated into Erlenmeyer flasks containing 99 mL of warm (48°C) sterile TSBYE and the contents of each flask were mixed with a magnetic stir bar. Flasks were heated with stirring, in a 48°C waterbath for 20 min. After heat shock treatment, the flask was immediately transferred to a 60°C waterbath and heated, with stirring, for 15 min. For enumeration of bacteria, 1.5 mL aliquots were removed from the flask after heat shock, and at 1, 2, 3, 5, 7, 9, 10, and 15 min intervals. The aliquots were put into 1.5 mL microcentrifuge tubes and kept in ice water until enumeration. Cells were serially diluted in sterile 0.1% peptone water (BD) and enumerated by spread plating on TSAYE. Petri plates were incubated at 30°C for 24 h prior to enumeration.

3.5.2 Acid tolerance

To determine the tolerance of *L. monocytogenes* to acid, the acid tolerance procedure of Aguilar (2004) was used. One mL of a 24 h subculture of each strain was separately inoculated into test tubes containing 9 mL of 0.1 M acetic acid (Fisher Scientific), pH 3.6. The samples were mixed by vortexing and incubated at 25°C for 25 h. Aliquots (1 mL) were removed every 5 hours and enumerated by spread plating on TSAYE. Petri plates were incubated at 30°C for 24 h prior to enumeration.

3.5.3 Salt tolerance

To determine the tolerance of *L. monocytogenes* to salt, a modified procedure of Aguilar (2004) was used. One mL of a 24 h subculture of each strain was separately inoculated into test tubes containing 9 mL of 0.1 M phosphate buffer (pH 7.0; Fisher Scientific) containing 8% NaCl (Fisher Scientific), or 0.1 M phosphate buffer alone to

ensure there was no antimicrobial effect from the buffer. The samples were mixed by vortexing and incubated at 25°C for 30 days. Aliquots (1 mL) were removed every 5 days and enumerated by spread plating on TSAYE. Petri plates were incubated at 30°C for 24 h.

3.6 Isolation of total RNA from *Listeria monocytogenes*

Total RNA was isolated from *E. coli* DH5 α , *L. monocytogenes* FS 15, and its three leu^R variants for use in RFDD analysis (section 3.7) and dot blot confirmation of the RFDD results (section 3.7.3). *L. monocytogenes* FS 15 was chosen because in preliminary studies (data not shown), the strain exhibited unusually high heat tolerance compared with other strains of *L. monocytogenes*. Additionally, in heat tolerance assays, one leu^R variant of this strain had a different D-value from the wild type, while the other two variants unexpectedly did not (see section 4.4.1).

The isolation was carried out using a protocol for the FastRNA[®] Pro Blue Kit (Qbiogene, Irvine, CA) modified by Gursky (2004). Bacteria were subcultured from a frozen (-80°C) culture and grown in TSBYE at 30°C for 24 h. One mL of the culture was removed and inoculated into 14 mL TSBYE and grown at 30°C to an OD₆₀₀ of 0.6. Ten mL of the culture was centrifuged in a 15 mL conical tube (BD) at 1500 x g (Allegra 25R centrifuge; Beckman Coulter) for 15 min at 4°C to pellet the cells. After decanting the supernatant, the pellet was resuspended by vortexing in 1 mL RNApro[™] solution. The 1 mL solution was transferred to a tube containing Lysing Matrix B (provided with the kit), and homogenized in a Mini Bead-Beater-8[™] (Biospec Products, Bartlesville, OK) for 3 min at maximum speed. The resulting mixture was centrifuged (Eppendorf 5417 C/R) at 12 000 x g for 5 min at 4°C, and the supernatant was transferred to a new microcentrifuge tube and incubated for 5 min at 25°C. Chloroform (300 μ L, Fisher Scientific) was added to the tube, the tube was vortexed for 10 sec and incubated for 5 min at 25°C. The tube was centrifuged (Eppendorf 5417 C/R) at 12 000 x g for 5 min at 4°C, and the upper phase was transferred to a new microcentrifuge tube. The RNA was precipitated by adding 500 μ L of 95% ethanol, inverting the tubes five times, and incubating the tube at

-20°C for 30 min. The RNA was pelleted by centrifugation at 12 000 x g for 15 min at 4°C, and the pellet was washed with 500 µL 75% ethanol. The ethanol was aspirated and the pellet was dried at 25°C. The RNA pellet was resuspended with 100 µL MilliQ water that had been treated with diethyl pyrocarbonate (DEPC) to remove RNases. After resuspension, the pellet was incubated for 5 min at 25°C, and mixed by flicking the tube. RNA was stored at -80°C until use.

The DNA-free™ kit (Ambion, Austin, TX) was used to remove contaminating DNA from the RNA preparations. In a 1.5 mL microcentrifuge tube, 25 µL of the RNA extraction was added to 14 µL DEPC-treated MilliQ water, 5 µL 10x DNase I buffer, and 0.5 µL recombinant DNase I (rDNase I). The reaction mixture was mixed gently by flicking and the tube was incubated at 37°C for 30 min. An additional 0.5 µL rDNase I was added to the tube and the tube was incubated for a further 30 min. To stop the reaction, 5 µL DNase Inactivation Reagent was added and the tube was incubated for 2 min at 25°C. The reagents were mixed every 30 sec by flicking. The inactivation reagent and the rDNase I were removed by centrifuging at 10 000 x g for 1.5 min at 4°C, and the RNA was transferred to a fresh tube and stored at -80°C until use.

Removal of contaminating DNA was verified by PCR using primers for the 16S rRNA gene (Tannock *et al.* 1999). The primers used were HDA1 (5'-ACT CCT ACG GGA GGC AGC AGT-3') and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA C-3'). Each reaction contained 1x PCR buffer, 3 mM MgCl₂, 100 µM of each dNTP, 0.4 µM of each primer, 1 µL template DNA, and 1.25 U Taq DNA polymerase (Invitrogen Canada). The reaction conditions consisted of a 4 min hot start at 94°C followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 4 min. Reactions were analysed with electrophoresis on a 1.5% agarose gel. A gene product of approximately 200 bp was indicative of DNA contamination. For each sample free of DNA, RNA concentration was determined using a GeneQuant Pro (Biospec, Cambridge, UK). RNA quality was determined by running 500 ng of DNA-free RNA on a 0.8% agarose gel at 5 Volts/cm for 90 min.

3.7 Restriction fragment differential display analysis of total RNA

To screen for differential gene expression among wild type and *leu^R* variants of *L. monocytogenes* FS 15, the displayPROFILE™ Kit for prokaryotic restriction fragment differential display (RFDD; Qbiogene) PCR was used, with some protocol modifications by Gao (2001). First- and second-strand synthesis of cDNA was performed on 1 µg total RNA in a 0.25 mL thin-walled PCR tube (Rose Scientific, Edmonton, AB). The first-strand synthesis was carried out at 42°C for 2 h in a GeneAmp PCR system 2400 thermocycler (Perkin Elmer, Norwalk, CT), and second-strand synthesis was performed at 16°C for 2 h. The cDNA was extracted with phenol (Invitrogen Canada) and chloroform. The aqueous phase was retained. The cDNA was precipitated by addition of 0.1 volume of 3 M sodium acetate (pH = 5.2) and 2 volumes of 95% ethanol, followed by overnight incubation at -20°C. The DNA was pelleted and washed with 70% ethanol. The pellet was air-dried and resuspended in sterile DEPC-treated MilliQ water. The cDNA was digested with *Taq* I endonuclease at 65°C for 2 h. Adaptors were ligated onto the *Taq* I sticky ends using T4 DNA ligase at 37°C for 3 h. The 0-extension Primer was end-labeled with [$\gamma^{33}\text{P}$]-ATP (3 000 Ci/mmol; Amersham Biosciences, Uppsala, Sweden) using T4 polynucleotide kinase at 37°C for 30 min, followed by deactivation of T4 kinase at 70°C for 5 min. Equal amounts of cDNA were used as template for PCR in 32 separate reactions, each containing one of the 32 DisplayPROFILE™ Probe Primers and the labeled 0-extension Primer. Reactions were carried out using touch-down PCR. Initial denaturation at 94°C for 1 min was followed by denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 1 min. The annealing temperature was reduced by 0.5°C each cycle for 10 cycles, with a final annealing temperature of 55°C. Amplification was continued for a further 25 cycles with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min. A final elongation was carried out at 72°C for 5 min.

After completion of PCR, loading buffer was added to each tube and the tube was incubated at 85°C for 5 min. Samples were kept on ice and loaded onto a 6% polyacrylamide sequencing gel (Bio-Rad) containing 8 M urea (EMD Pharmaceuticals,

Durham, NC) that was pre-run for 30 min at 40 Watts. The electrophoresis buffer was 0.6x TBE (Tris-borate-EDTA) and the gel was run at 40 Watts until the bromophenol blue had run off the bottom of the gel and the xylene cyanol was midway to two-thirds down the gel. The gel was transferred to Whatman paper (Whatman, Middlesex, UK), dried (Model 583 Gel Dryer, Bio-Rad) and exposed to X-Omat AR film (Eastman Kodak, Rochester, NY) for 12 to 24 h. Intensity of bands in the autoradiograms were analyzed by densitometry using a GS-670 imaging densitometer (Bio-Rad).

Statistical analysis of band intensity was performed in Excel (Microsoft Corporation, Redmond, WA) using a two-tailed T-test. The mean intensity of the band in the three leu^R samples was compared with the intensity of the band in the wild type sample. Significance was determined at the 95% confidence level. Bands that had significant differences in intensity were located on the gel by re-alignment with the autoradiogram and marked using a 25G5/8 syringe needle (BD) to make a hole through the x-ray film and the gel. The band was excised from the gel with a sterile scalpel, and the gel slice was then placed in a 1.5 mL microcentrifuge tube and heated at 95°C for 15 min in 50 µL TE buffer. The gene fragment was amplified by PCR according to the protocol in the displayPROFILE™ RFDD Kit manual, using the appropriate Probe primer. A 10 µL sample of the PCR product was then run on a 1.5% agarose gel to verify amplification of the gene fragment.

3.7.1 Cloning of differentially expressed fragments

Each differentially expressed gene fragment was cloned into the vector pCR®2.1 by TA cloning (TA Cloning® Kit; Invitrogen Canada). This technique takes advantage of the single 3'-deoxyadenosine (A) overhang that is added to PCR products by *Taq* polymerase. The PCR product can easily be ligated into a vector in its linearized form if that vector, such as pCR®2.1, contains a single 3'-deoxythymidine (T) overhang at each end. A plasmid map of the circularized form of pCR®2.1 is shown in Figure 1, illustrating where insertion of the PCR product into the vector can occur.

E. coli TOP10 cells (Invitrogen Canada) were transformed with pCR®2.1 containing a differentially expressed gene fragment as an insert. Transformants were

selected by growth on LB agar containing 50 µg/mL Km and 40 µL of 40 mg/mL X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside; Invitrogen Canada). Blue colonies were picked for each clone and grown in LB broth containing 50 µg/mL Km for 16 h at 37°C with aeration. Cultures were stored in LB broth and 20% glycerol at -80°C.



Figure 1: Plasmid map of vector pCR®2.1 (Invitrogen Canada)

3.7.2 Plasmid isolation and amplification of inserts

Plasmids containing the cloned fragments were isolated from the transformed *E. coli* strains using miniprep plasmid isolation by alkaline lysis with SDS (Sambrook and Russel 2001). To confirm the presence of an insert in the original vector,

the primers M13 Rev (5'-CAG GAA ACA GCT ATG AC-3') and T7 Pro (5'-TAA TAC GAC TCA CTA TAG GG-3') were used to amplify the inserts from the pCR[®]2.1 constructs (Invitrogen Canada). Amplification was carried out with a 5 min hot start at 94°C, followed by 35 cycles of 94°C for 45 sec, 45°C for 30 sec, and 72°C for 30 sec, and extension at 72°C for 10 min. A 10 µL aliquot of each reaction was separated on a 1.5% agarose gel containing 0.5 mg/mL EtBr at 5 V/cm for 90 min. A standard curve was constructed using the migration distances of each of the bands in the 100 bp ladder. The sizes of the amplicons, which consist of the insert plus 176 bp of flanking sequence from pCR[®]2.1, were determined to calculate the size of each insert.

3.7.3 Dot blot confirmation of differential expression

Confirmation of differential expression of gene fragments isolated from RFDD was performed with a dot blot procedure, using a protocol modified from Bibiloni *et al.* (2005). Total RNA from *E. coli* DH5α, *L. monocytogenes* FS 15, FS 15L1, FS 15 L2, and FS 15L3 was denatured for 10 min at 25°C in 2% glutaraldehyde (Sigma-Aldrich) in 4 mM sodium phosphate (VWR). RNA was initially diluted to 2 µg/mL with RNase-free MilliQ water containing 0.001% bromophenol blue. Using the Bio-Dot Apparatus (Bio-Rad), two-fold serial dilutions of RNA (starting at 200 ng) were applied to pre-wet Hybond N⁺ membranes (Amersham) in duplicate. The denatured RNA was fixed to the membranes in 0.05 M NaOH (VWR) with gentle agitation on a Maxi Rotator rotary shaker (Barnstead International, Dubuque, IA) and washed with agitation in 2x SSC (3 M NaCl, 0.3 M sodium citrate, pH = 7.0). Membranes were stored in plastic wrap at 4°C until hybridization.

A total of five probes were used for hybridization experiments, consisting of the four gene fragments from RFDD analysis, and Eub 338 (5'-GCT GCC TCC CGT AGG AGT-3') which recognizes the 16S rRNA of all eubacteria (Amann *et al.* 1990).

The RFDD probes were prepared by PCR amplification using the procedure in section 3.7.2. After running a gel to verify the amplification, the remaining 40 µL of reaction product was cleaned using the QIAquick PCR Purification Kit (Qiagen, Venlo,

Netherlands). The clean PCR products were digested with *Eco*RI (Invitrogen Canada) to remove flanking sequences from the vector. After 1 h, digests were immediately placed on ice and then separated on a 1.5% agarose gel containing 0.5 mg/mL EtBr at 5 V/cm for 90 min. Under UV light, bands corresponding to the size of each insert were excised with a sterile scalpel. The DNA was removed from the agarose using the QIAEX II Gel Extraction Kit (Qiagen) and then stored at -20°C until labeling. Probes were labeled by incorporation of [α -³²P] dCTP using the Random Primers DNA Labeling System (Invitrogen Canada).

The probe Eub 338 was synthesized by the DNA CORE lab (Department of Biochemistry, University of Alberta). Eub 338 was labeled by terminal phosphate exchange with [γ -³²P]ATP using T4 polynucleotide kinase (Invitrogen Canada).

For hybridization with Eub 338, the membrane was incubated at 42°C for 2 h in 10 mL hybridization buffer (1 M NaCl, 5 mM sodium phosphate buffer, 5 mM EDTA, 0.5% SDS, 1x Denhardt's solution). After pre-hybridization, labeled Eub 338 was added and the membrane was left to incubate for 24 h at 42°C. The membrane was washed twice at 59°C for 30 min using 50 mL washing solution (1x SSC, 0.5% SDS). The membrane was exposed to X-ray film (Eastman Kodak) for 16 to 24 h.

For hybridization with the RFDD probes, the hybridization conditions of Gao (2001) were used. Membranes were incubated at 65°C for 2 h in 10 mL hybridization buffer (6x SSPE, 0.5% SDS, 5x Denhardt's solution). After pre-hybridization, the labeled RFDD probe was added and the membranes left to incubate for 24 h at 65°C. Membranes were washed twice for 10 min at 65°C using 50 mL washing solution A (2x SSPE, 0.1% SDS) and once for 30 min at 65°C using 50 mL washing solution B (0.1x SSC, 0.1% SDS). Membranes were exposed to X-ray film (Eastman Kodak) for 16 to 24 h.

Densitometry measurements were made using the Quantity One 1-D Analysis software (Bio-Rad). A standard curve was constructed using signal intensities from hybridization with Eub 338. The samples of *E. coli* DH5 α RNA were used as standards, and the amount of RNA in the FS15 dots were interpolated from the standard curve. The overall signal intensity for each dot was measured as a ratio of intensity from hybridization with the unknown gene fragment and intensity from hybridization with Eub

338. A two-tailed T-test was used to compare the ratio of the wild type FS 15 sample with all three leu^R variants and determine the significance at the 95% confidence interval.

3.8 Sequencing of differentially expressed fragments

The sequences of differentially expressed gene fragments were determined using the BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The gene fragments were amplified by PCR using high fidelity Platinum Taq DNA polymerase (Invitrogen Canada). Each reaction contained 1x high fidelity PCR buffer, 200 μ M of each dNTP, 2 mM MgSO₄, 0.2 μ M primer T7 Pro, 0.2 μ M primer M13 Rev, 10 pg plasmid template, and 1 U Platinum Taq. The PCR program consisted of 2 min incubation at 94°C followed by 35 cycles of 94°C for 30 sec, 45°C for 30 sec, and 68°C for 1 min, with a final extension phase at 72°C for 10 min. The product was cleaned with the QIAquick PCR Purification Kit (Qiagen) and used as template for the sequencing reaction. BigDye premix was added to 0.5x buffer (200 mM Tris, pH 9.0; 5 mM MgCl₂), 5 pmol M13 Reverse primer, and 300 ng template DNA. The reaction mixture was then run for 25 cycles in a thermocycler (Applied Biosystems) using the following program: 96°C for 30 sec, 50°C for 15 sec, and 60°C for 2 min. The reaction was cleaned and de-salted with NaOAc/EDTA, washed with ethanol, and air-dried at 25°C. Sequencing of the DNA was completed on an AB-373 DNA sequencer (Applied Biosystems) by the Molecular Biology Services Unit at the University of Alberta. The gene fragments were identified by homology with known sequences using a nucleotide-nucleotide BLAST search (<http://www.ncbi.nlm.nih.gov/>) available from the National Center for Biotechnology Information (NCBI).

4 Results

4.1 Bacteriocin purification

Partially purified leucocin A was required for creating leu^R strains of *L. monocytogenes*. Leucocin A was concentrated from the culture supernatant of *L. gelidum* UAL187, and a spot-on-lawn assay showed that 6 400 AU/mL of partially purified leucocin A was recovered.

4.2 Isolation of leucocin-resistant *Listeria monocytogenes*

Three leu^R colonies for each strain of *L. monocytogenes* ATCC 15313, CDC 7762, and FS 15 were picked from agar plates containing 200 AU/mL leucocin A. These strains were subcultured for four consecutive days in TSBYE in the absence of the selective pressure of leucocin A to determine the stability of the resistance characteristic. A deferred inhibition assay after the fourth subculture indicated that all 9 leu^R variants were still resistant to leucocin A, while the three wild types were still susceptible. Small isolated colonies were observed within the inhibition zones of the wild type overlays, which indicated the presence of leu^R cells. The 9 leu^R variants were selected for further study.

In a spot-on-lawn assay, none of the 9 leu^R variants were inhibited by 10 µL of 6 400 AU/mL leucocin A, and they were able to form confluent lawns with no zones of inhibition. Wild type *L. monocytogenes* ATCC 15313, CDC 7762, and FS 15 were inhibited by all spots containing > 100 AU/mL leucocin A.

4.3 RAPD analysis of leucocin-resistant *Listeria monocytogenes*

Randomly amplified polymorphic DNA analysis was performed to confirm that the leu^R variants had the same profile as the wild type parent strains, and therefore were descended from them. Within each of the three strains, the RAPD profile observed with

primer OPM-01 was identical for the wild type and the *leu*^R variants. Similar results were observed with primer M13. Furthermore, each of the three strains had a unique banding pattern. None of the *leu*^R variants had RAPD profiles that were different from their parent strain. Figure 2a shows the RAPD profiles generated using primer M13 for *L. monocytogenes* strain CDC 7762 and its *leu*^R variants, with 4 bands at ~950 bp, 1 400 bp, ~1 580 bp, and ~1 700 bp. *L. monocytogenes* FS 15 had a very different profile with a total of 5 bands at 1 050 bp, 1 400 bp, 1 600 bp, 2 000 bp, and ~3 500 bp. Similarly, in Figure 2b, RAPD profiles determined using primer OPM-01 for *L. monocytogenes* CDC 7762 had 4 bands at 400 bp, 1 500 bp, 1 900 bp, and < 3 530 bp. Again, *L. monocytogenes* FS 15 had a very different RAPD profile, with 2 bands at 950 bp and 1 400 bp, and two bands between 2 072 and 3 530 bp.

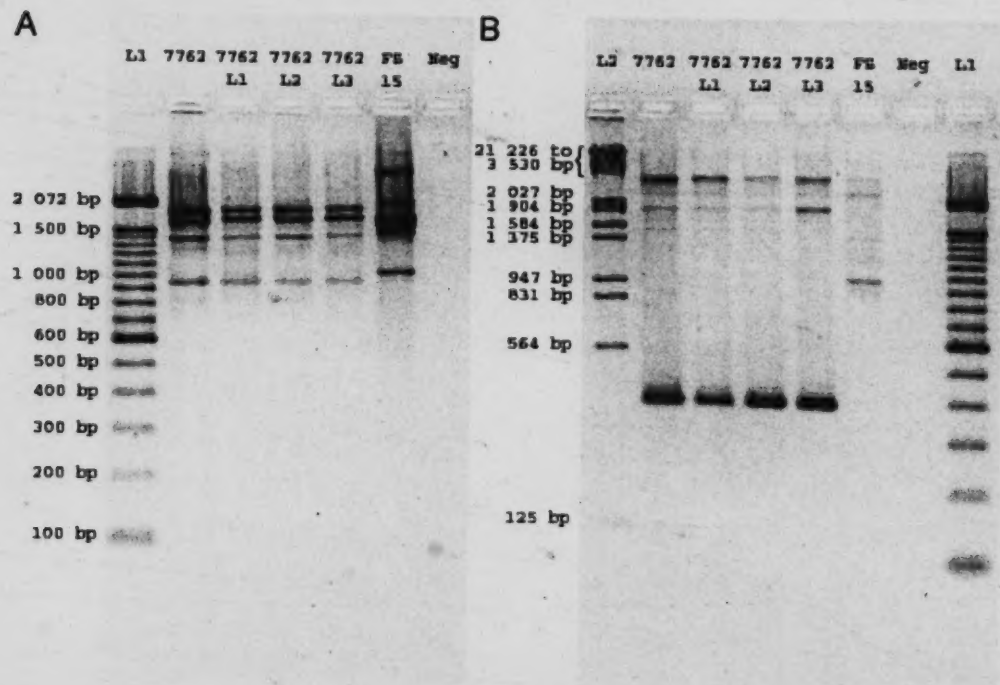


Figure 2: Agarose gel of RAPD profiles for wild type *L. monocytogenes* FS 15, CDC 7762, and the leucocin-resistant CDC 7762L1, CDC 7762L2, and CDC 7762L3 using primers M13 (A) and OPM-01 (B)
L1 = 100 bp ladder, L2 = λ DNA (*Eco* RI, *Hind* III digest), Neg = negative control

4.4 Stress tolerance of leucocin-resistant *Listeria monocytogenes*

The leucocin-susceptibility of wild type *L. monocytogenes* ATCC 15313, CDC 7762, FS 15, and the leu^R variants was tested by deferred inhibition assay prior to use in stress tolerance assays. This was done to ensure that the wild type strains were still susceptible to leucocin A, and the leu^R variants had not reverted to the wild type phenotype. Moderately-sized zones of inhibition (1 to 3 cm in diameter) caused by a proteinaceous substance were visible for the wild type *L. monocytogenes*. No zones of inhibition were observed for the leu^R variants, but occasionally there were small (< 1 cm) zones of inhibition that were not affected by pronase E. Therefore, each stress tolerance assay was performed with wild type strains that were susceptible to leucocin A, and their leu^R variants.

4.4.1 Heat tolerance

The survival of *L. monocytogenes* in TSBYE at 60°C for 15 min was assayed to determine the effect of leucocin resistance on the heat tolerance of leu^R cultures. Survival curves for the wild type and leu^R variants of all three *L. monocytogenes* strains are shown in Figures 3 to 5. In Figure 3, the survival curves of wild type and leu^R *L. monocytogenes* ATCC 15313 are very similar, with consistency among replicates. There was a lot of variability among the replicate survival curves for *L. monocytogenes* CDC 7762 shown in Figure 4. In Figure 4a, replicate 3 of the wild type strain had a 2 log drop in population in the first minute of the experiment, before the death rate decreased to resemble that observed in the other two replicates. The D-value for this curve was calculated from the second linear portion of the curve, and not the initial decrease. Figure 4b shows variation among replicates for survival curves of variant CDC 7762L1 as the cell populations begin to diverge after 7 min. Figure 4c also shows variability among replicates with variant CDC 7762L2, but it is only apparent after 10 min. In Figure 5, there is little variability among replicates for strain FS 15. The leu^R variants have a 0.5 log decrease, but the drop in the wild type strain ranges from 0.5 to 1 log, so the survival curves might not be different.

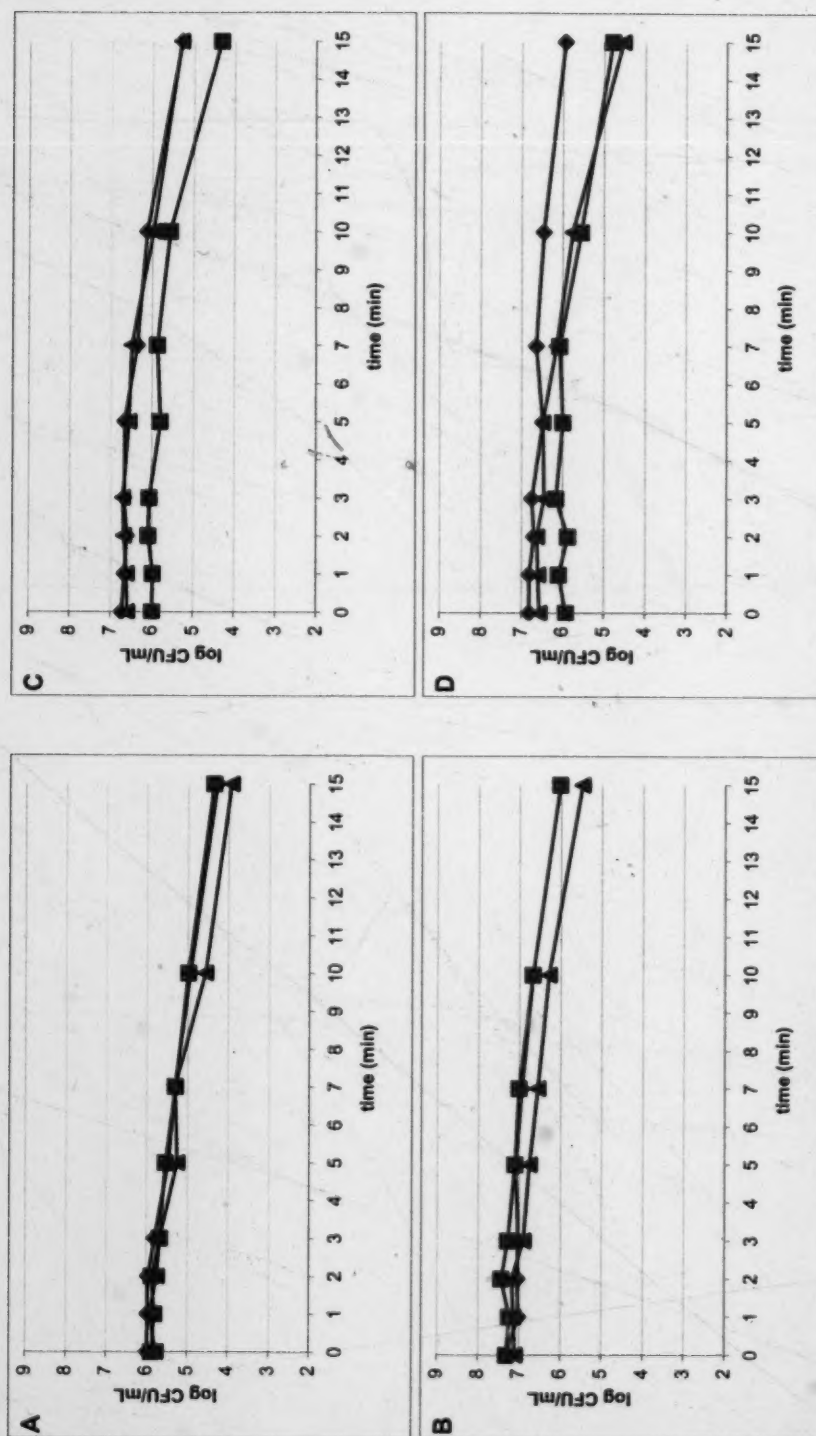


Figure 3: Survival of *Listeria monocytogenes* heated in TSBYE for 15 min at 60°C. Strains examined are wild type *Listeria monocytogenes* ATCC 15313 (A) and leucocin-resistant variants ATCC 15313L1 (B), ATCC 15313L2 (C), and ATCC 15313L3 (D). Experiments were performed in triplicate; ▲ = Replicate 1, ■ = Replicate 2, ◆ = Replicate 3

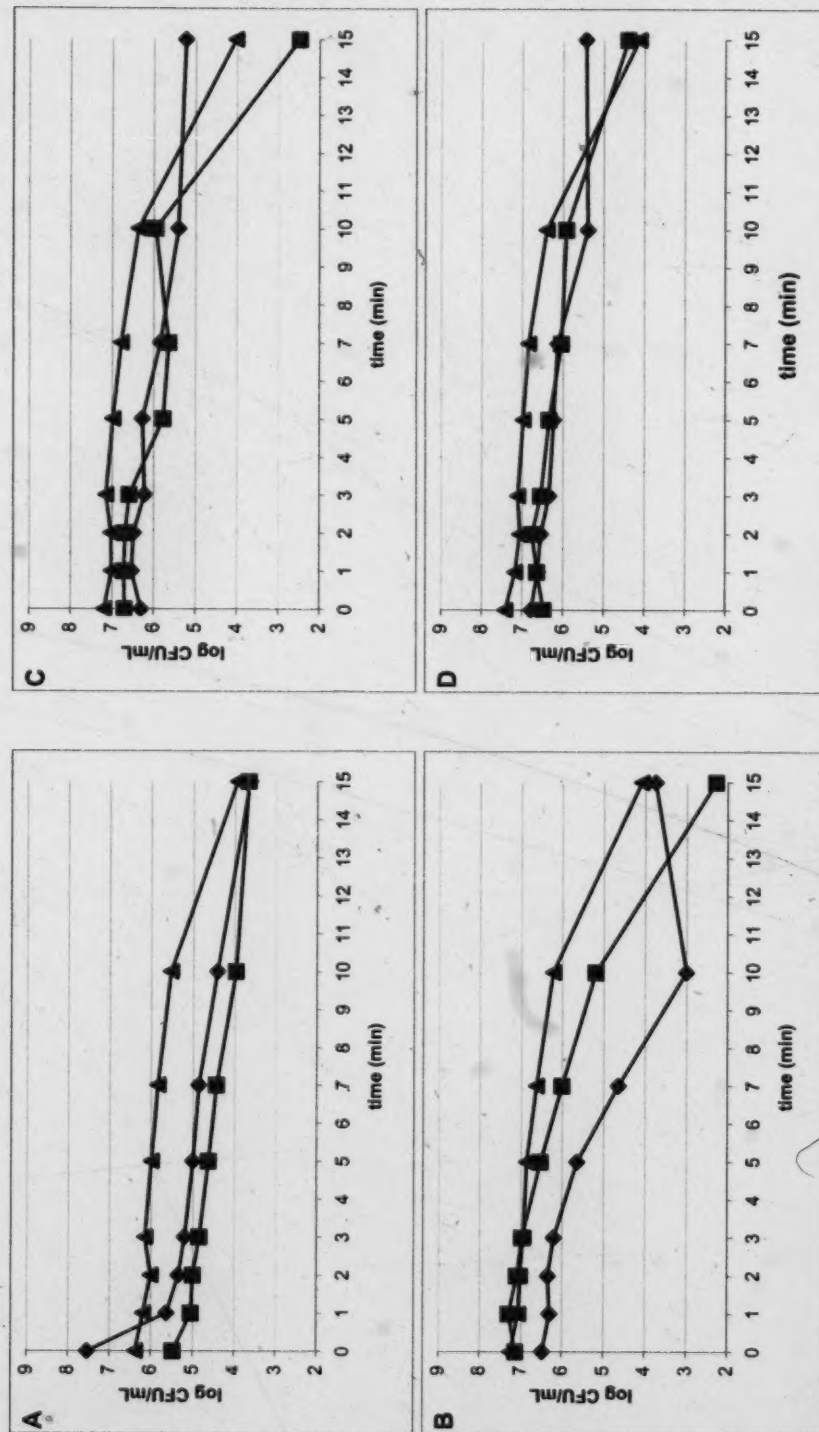


Figure 4: Survival of *Listeria monocytogenes* heated in TSBYE for 15 min at 60°C. Strains examined are wild type *Listeria monocytogenes* CDC 7762 (A) and leucocin-resistant variants CDC 7762L1 (B), CDC 7762L2 (C), and CDC 7762L3 (D). Experiments were performed in triplicate; ▲ = Replicate 1, ■ = Replicate 2, ◆ = Replicate 3

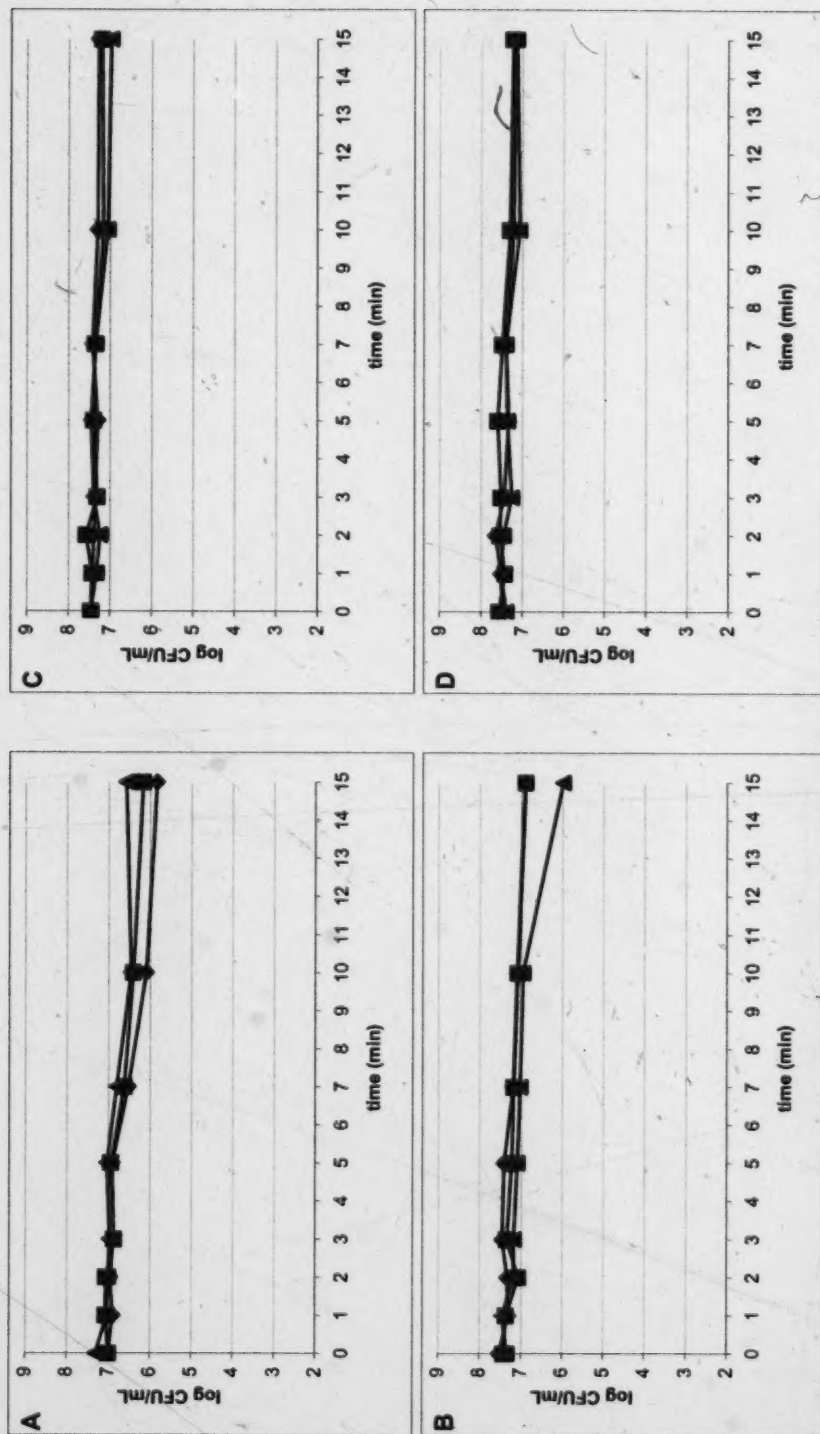


Figure 5: Survival of *Listeria monocytogenes* heated in TSBYE for 15 min at 60°C. Strains examined are wild type *Listeria monocytogenes* FS 15 (A) and leucocin-resistant variants FS 15L1 (B), FS 15L2 (C), and FS 15L3 (D). Experiments were performed in triplicate; ▲ = Replicate 1, ■ = Replicate 2, ◆ = Replicate 3

The heat tolerance of the wild type and leu^R variants is represented by the D-value, which is defined as the amount of time required for a one log reduction in the bacterial population. The D-values for each of the strains of *L. monocytogenes* and their leu^R variants are shown in Table 3. The D-values of the wild type strains were compared to determine if there were significant differences in the tolerance of the strains to heat. No significant difference ($P > 0.05$) among the D-values of the wild type *L. monocytogenes* was found. The D-values for leu^R *L. monocytogenes* ATCC 15313 were not significantly different ($P > 0.05$) from the D-value of the wild type. Similar results were observed for *L. monocytogenes* CDC 7762. The D-value for wild type *L. monocytogenes* FS 15 was similar to those obtained for *L. monocytogenes* FS 15L1 and FS 15L3 ($P > 0.05$), but it was significantly lower ($P < 0.05$) than the D-value of *L. monocytogenes* FS 15L2. The D-value of FS 15L2 was not significantly different from the D-values of *L. monocytogenes* FS 15L1 and FS 15L3 ($P > 0.05$).

Table 3: D-values for heat tolerance assays of wild type and leucocin-resistant *Listeria monocytogenes* ATCC 15313, CDC 7762, and FS 15

Strain		Average D-value ^a (min)	P value	SEM ^b	Duncan Grouping
ATCC 15313	wild type	7.5	0.770	1.0	A
	L1	8.0			A
	L2	6.5			A
	L3	7.6			A
CDC 7762	wild type	7.9	0.385	2.0	A
	L1	2.9			A
	L2	6.3			A
	L3	6.1			A
FS 15	wild type	12.6	0.040	5.5	B
	L1	26.9			AB
	L2	40.6			A
	L3	31.1			AB

^aEach D-value is the mean of three replicates

^bStandard error of the means

4.4.2 Acid tolerance

The survival of *L. monocytogenes* in 0.1 M acetic acid (pH = 3.6) for 25 h was assayed to determine if leucocin resistance had any effect on acid tolerance. The survival curves for wild type and leu^R variants of *L. monocytogenes* ATCC 15313, CDC 7762, and FS 15 held in 0.1 M acetic acid are shown in Figures 6 to 8. Figure 6 shows the survival curves for strain ATCC 15313. There was some variability among replicates, and the survival curves for the leu^R variants were steeper than that of the wild type, with a 3 log decrease over 25 h as opposed to 2 logs for the wild type. Data in Figure 7 demonstrate that the leu^R variants of *L. monocytogenes* CDC 7762 had steeper survival curves than the wild type strain. There was a slight (< 1 log) decrease in the population of the wild type strain, but the leu^R variants decreased in population by up to 1.5 log units. The survival curves of *L. monocytogenes* FS 15 had little variability among replicates, but there was one outlier at the 10 min point for replicate 3 for the leu^R variant FS 15L3. There was variability in the steepness of the survival curves for the leu^R variants, and the drop in population ranged from 0.5 log units to just over 1 log unit.

The D-values calculated from the survival curves for acid tolerance of wild type and leu^R *L. monocytogenes* are shown in Table 4. The D-values for acid tolerance of the wild type strains were compared to determine if there were significant differences, and no significant difference ($P > 0.05$) in D-value among the three wild type strains of *L. monocytogenes* was found. The D-values of leu^R variants of *L. monocytogenes* ATCC 15313 were similar to each other, and all three were significantly lower ($P < 0.05$) than the D-value of the wild type. Similarly, the D-values of the leu^R variants of *L. monocytogenes* CDC 7762 were significantly lower ($P < 0.05$) than the D-values of the wild type. No significant difference was found among the D-values for *L. monocytogenes* FS 15 and its leu^R variants ($P > 0.05$).

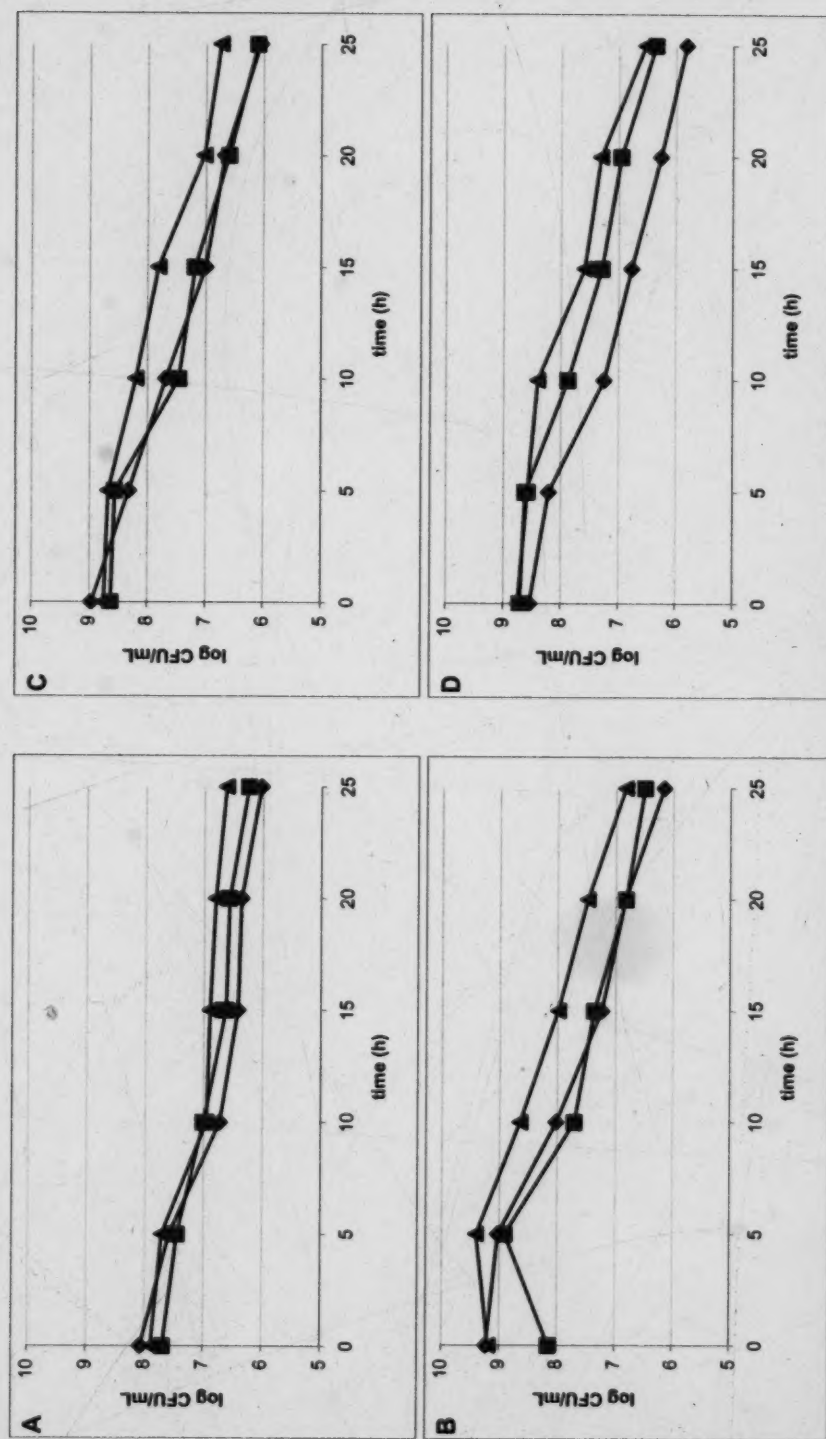


Figure 6: Survival of *Listeria monocytogenes* incubated in 0.1 M acetic acid, pH 3.6 at 25°C for 25 h. Strains examined are wild type *Listeria monocytogenes* ATCC 15313 (A) and leucocin-resistant variants ATCC 15313L1 (B), ATCC 15313L2 (C), and ATCC 15313L3 (D). Experiments were performed in triplicate; Δ = Replicate 1, $\square$ = Replicate 2, $\diamond$ = Replicate 3

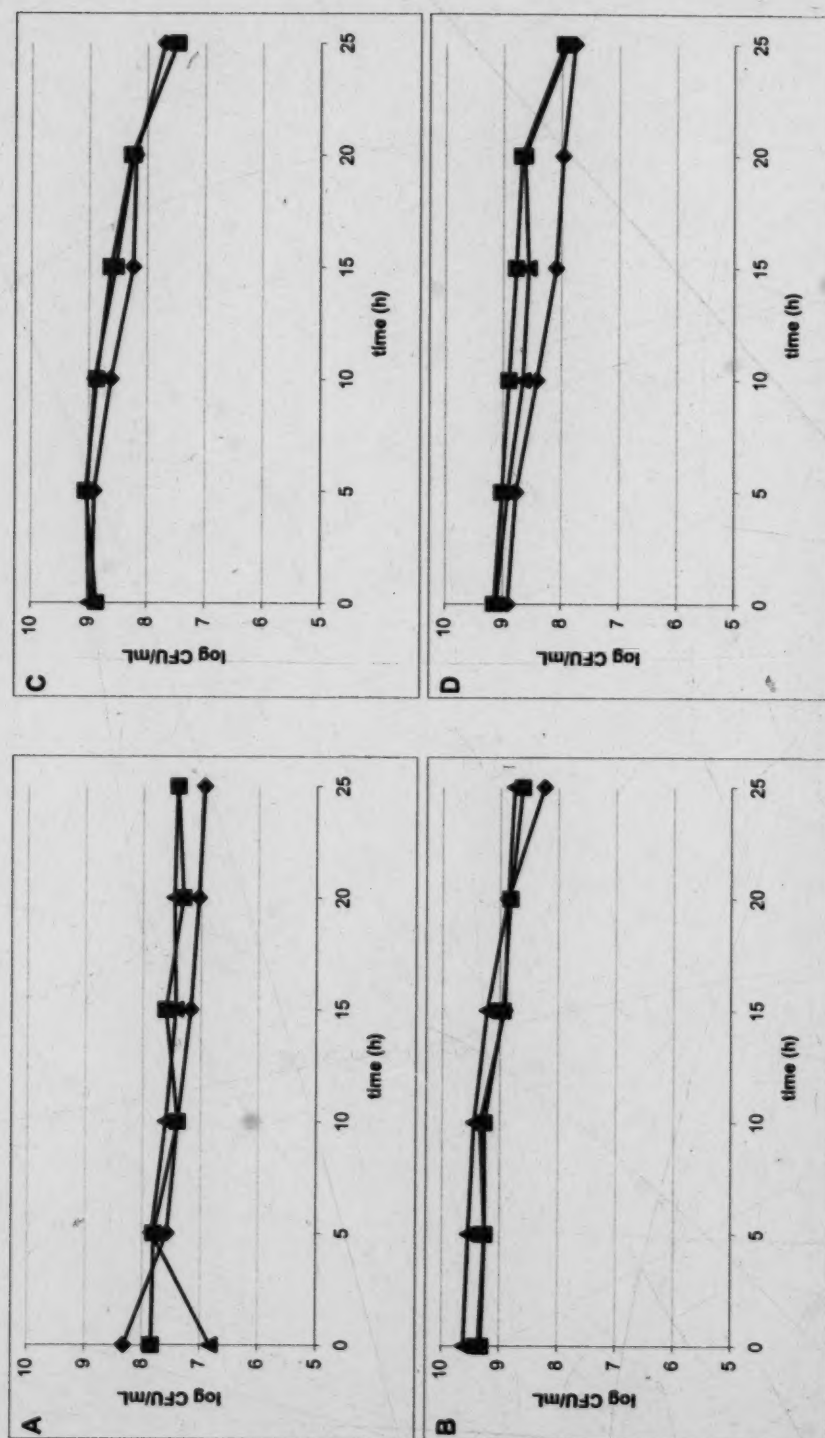


Figure 7: Survival of *Listeria monocytogenes* incubated in 0.1 M acetic acid, pH 3.6 at 25°C for 25 h. Strains examined are wild type *Listeria monocytogenes* CDC 7762 (A) and leucocin-resistant variants CDC 7762L1 (B), CDC 7762L2 (C), and CDC 7762L3 (D). Experiments were performed in triplicate; ▲ = Replicate 1, ■ = Replicate 2, ◆ = Replicate 3

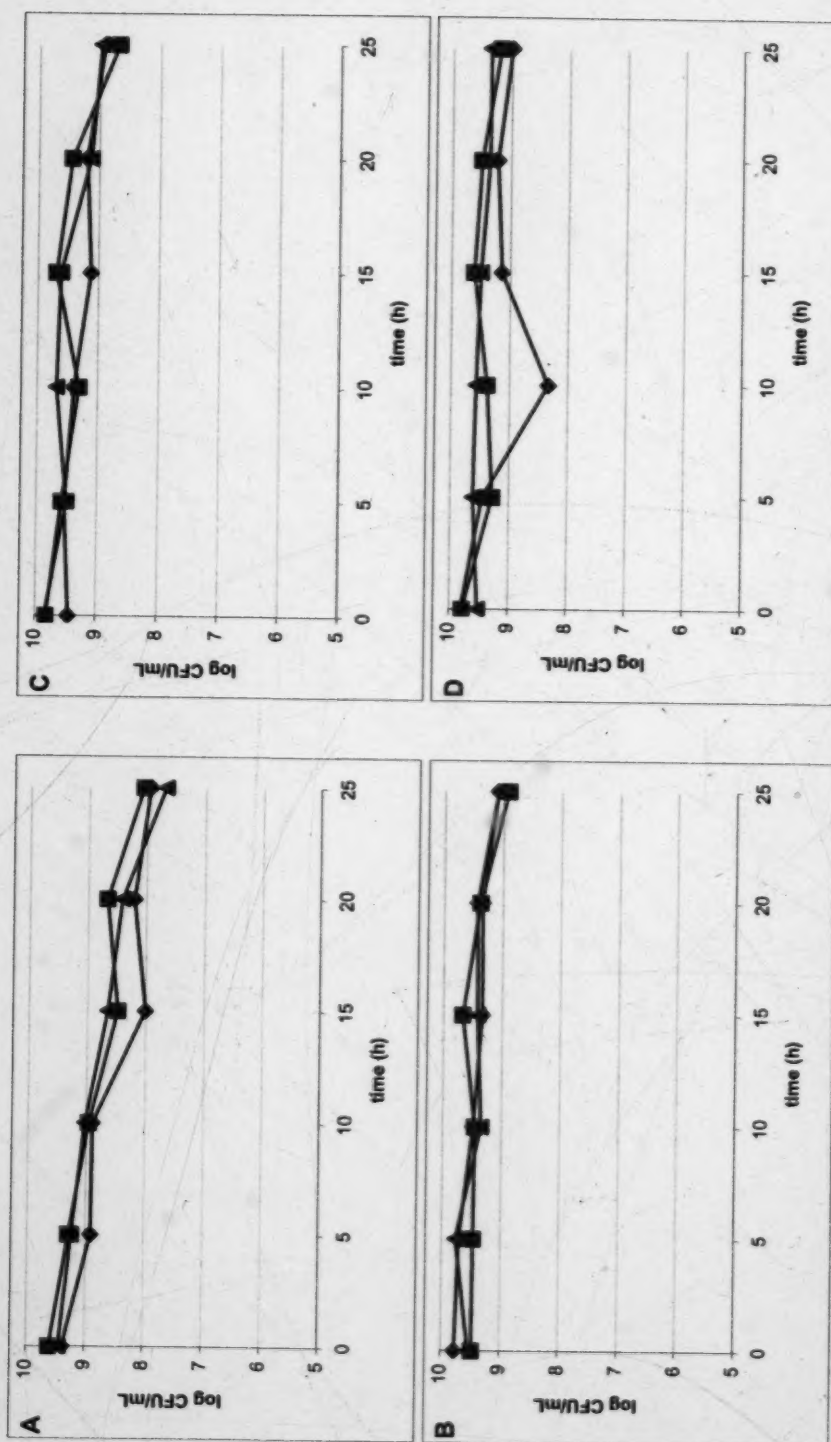


Figure 8: Survival of *Listeria monocytogenes* incubated in 0.1 M acetic acid, pH 3.6 at 25°C for 25 h. Strains examined are wild type *Listeria monocytogenes* FS 15 (A) and leucocin-resistant variants FS 15L1 (B), FS 15L2 (C), and FS 15L3 (D). Experiments were performed in triplicate; ▲ = Replicate 1, ■ = Replicate 2, ◆ = Replicate 3

Table 4: D-values for acid tolerance assays of wild type and leucocin-resistant *Listeria monocytogenes* ATCC 15313, CDC 7762, and FS 15

Strain		Average D-value ^a (h)	P value	SEM ^b	Duncan Grouping
ATCC 15313	wild type	29.7	0.036	4.7	A
	L1	9.0			B
	L2	9.9			B
	L3	9.5			B
CDC 7762	wild type	51.0	0.008	6.2	A
	L1	19.0			B
	L2	11.5			B
	L3	18.6			B
FS 15	wild type	16.9	0.178	8.1	A
	L1	29.3			A
	L2	19.6			A
	L3	42.7			A

^aEach D-value is the mean of three replicates

^bStandard error of the means

4.4.3 Salt tolerance

The survival of *L. monocytogenes* in 0.1 M phosphate buffer containing 8% NaCl and held at 25°C for 30 d was assayed to determine the effect of leucocin resistance on the salt tolerance of leu^R cultures. Inoculated tubes containing only 0.1 M phosphate buffer were included in each replicate to ensure that any antimicrobial effects were due to the 8% saline and not the buffer itself.

The survival curves for wild type and leu^R variants of *L. monocytogenes* ATCC 15313, CDC 7762, and FS 15 are shown in Figures 9 to 11. The survival curves for cultures held in 8% NaCl remained constant after an initial decrease of as much as a two log drop in population during the first 18 d. The population of wild type *L. monocytogenes* CDC 7762 increased by approximately one log after six days and then stabilized (Figure 10). This phenomenon was not observed in the survival curves for the other cultures.

There was no antimicrobial effect observed in cultures in phosphate buffer after 30 d. D-values could not be calculated because the survival curves remained constant over the 30 d of incubation. For some replicates, the bacterial population was up to one log higher after day 6 compared with day 0.

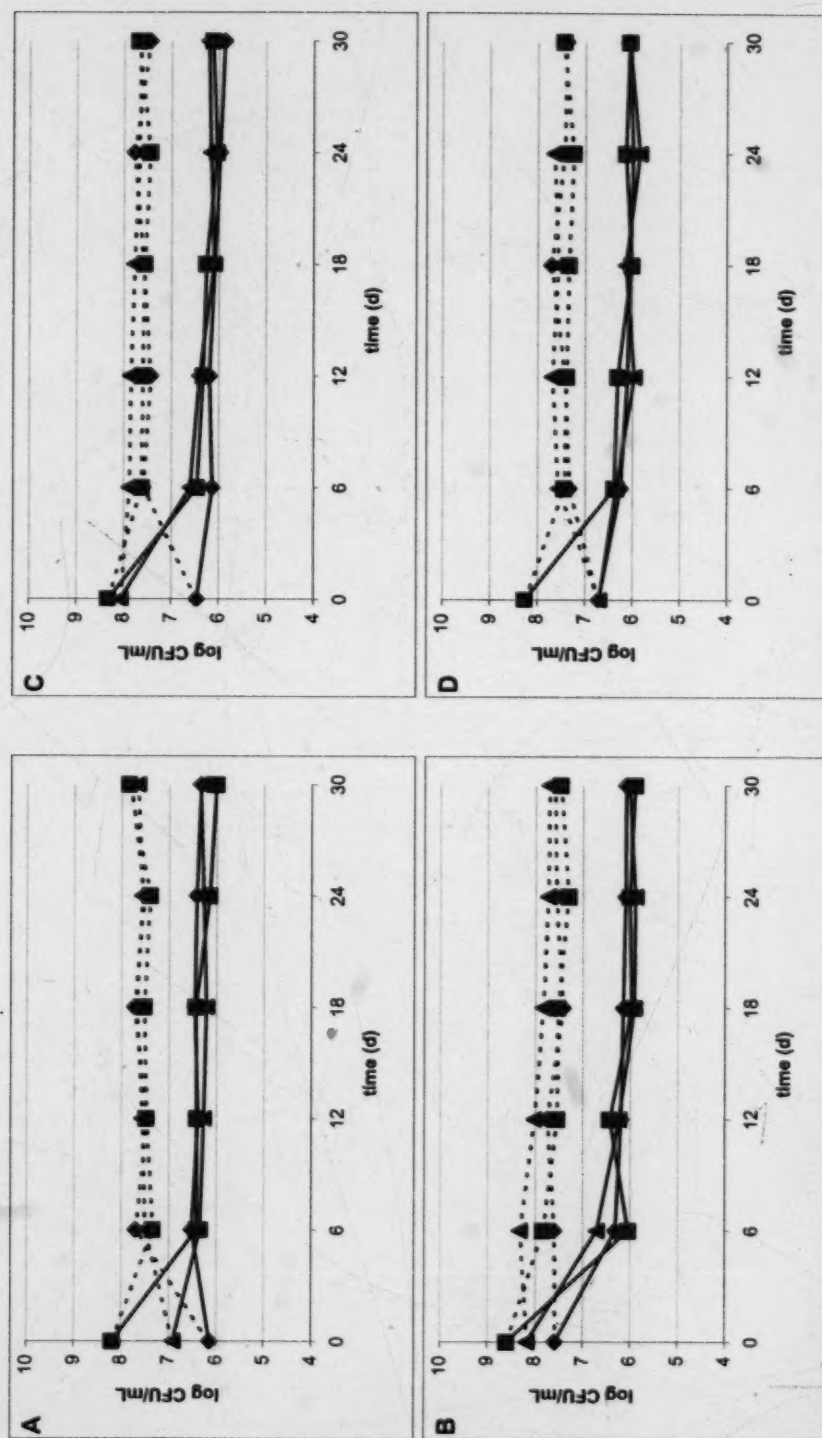


Figure 9: Survival of *Listeria monocytogenes* in 0.1 M phosphate buffer (pH 7.0) containing 8% NaCl for 30 d. Strains examined are wild type *Listeria monocytogenes* ATCC 15313 (A) and leucocin-resistant variants ATCC 15313L1 (B), ATCC 15313L2 (C), and ATCC 15313L3 (D). Experiments were performed in triplicate; Δ = Replicate 1, $\square$ = Replicate 2, $\diamond$ = Replicate 3. Parallel experiments performed solely in 0.1 M phosphate buffer (pH 7.0) are shown in hashed lines.

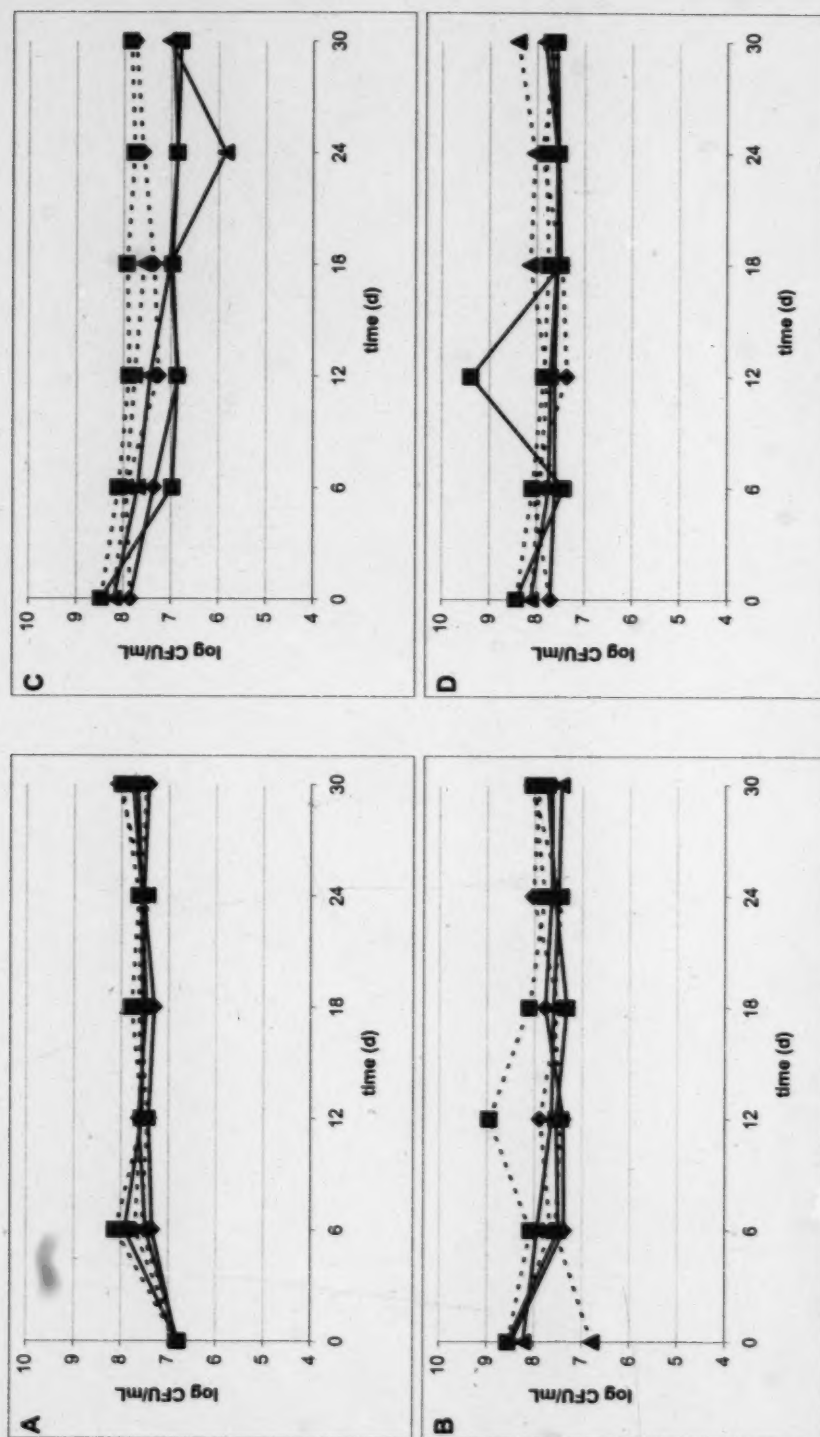


Figure 10: Survival of *Listeria monocytogenes* in 0.1 M phosphate buffer (pH 7.0) containing 8% NaCl for 30 d. Strains examined are wild type *Listeria monocytogenes* CDC 7762 (A) and leucocin-resistant variants CDC 7762L1 (B), CDC 7762L2 (C), and CDC 7762L3 (D). Experiments were performed in triplicate; Δ = Replicate 1, $\square$ = Replicate 2, $\diamond$ = Replicate 3. Parallel experiments performed solely in 0.1 M phosphate buffer (pH 7.0) are shown in hashed lines.

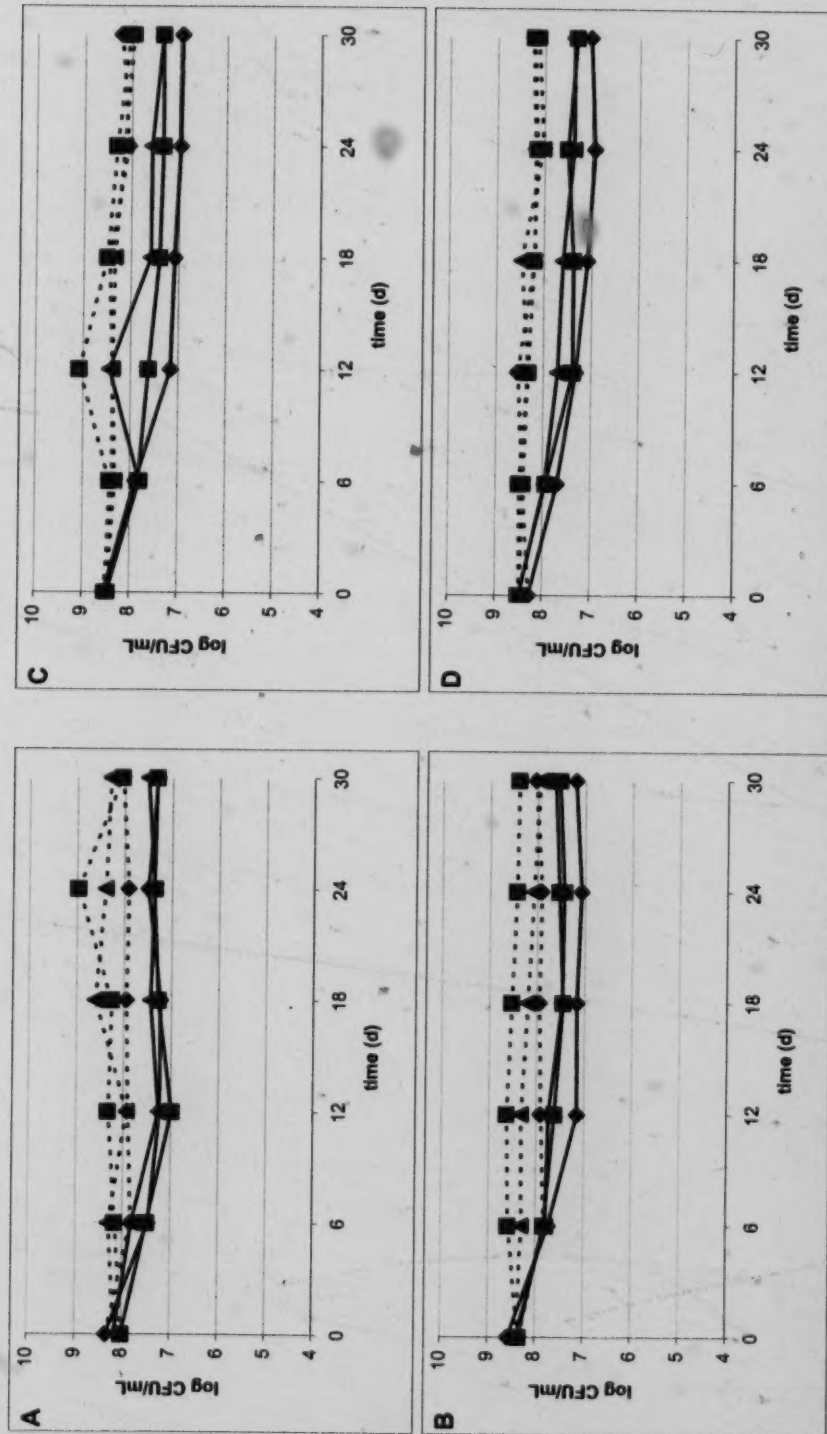


Figure 11: Survival of *Listeria monocytogenes* in 0.1 M phosphate buffer (pH 7.0) containing 8% NaCl for 30 d. Strains examined are wild type *Listeria monocytogenes* FS 15 (A) and leucocin-resistant variants FS 15L1 (B), FS 15L2 (C), and FS 15L3 (D). Experiments were performed in triplicate; Δ = Replicate 1, $\square$ = Replicate 2, $\diamond$ = Replicate 3. Parallel experiments performed solely in 0.1 M phosphate buffer (pH 7.0) are shown in hashed lines.

4.5 Isolation of total RNA from *Listeria monocytogenes*

Total RNA was isolated from *L. monocytogenes* FS 15 and its leu^R variants for analysis using RFDD. *L. monocytogenes* FS 15 and its leu^R variants were chosen for RFDD-PCR analysis because of the variation in the D-values of the leu^R strains for heat tolerance. Figure 12 shows an agarose gel of total RNA isolated from *L. monocytogenes* FS 15 and its leu^R variants. Bands visible on the gel indicate the positions of 23S, 16S, and 5S ribosomal RNA, and mRNA smears above and below the 23S and 16S bands. No degradation of RNA was evident.

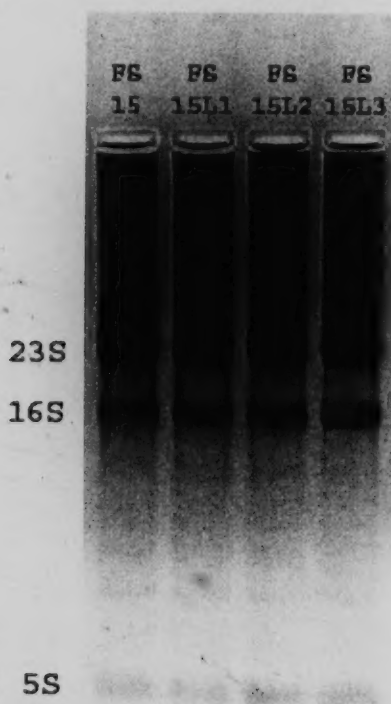


Figure 12: Agarose gel of total RNA isolated from wild type *Listeria monocytogenes* FS 15 and leucocin-resistant FS 15L1, FS 15L2, and FS 15L3

To ensure that DNA was not present in the isolated RNA, PCR was performed using the HDA primers and the resulting agarose gel is shown in Figure 13. The 200 bp product was present in the positive control containing DNA from *Listeria innocua* ATCC 33090. No amplicon was present in the rDNase-treated RNA samples, indicating the absence of DNA contamination. Unused primers were visible below 100 bp.

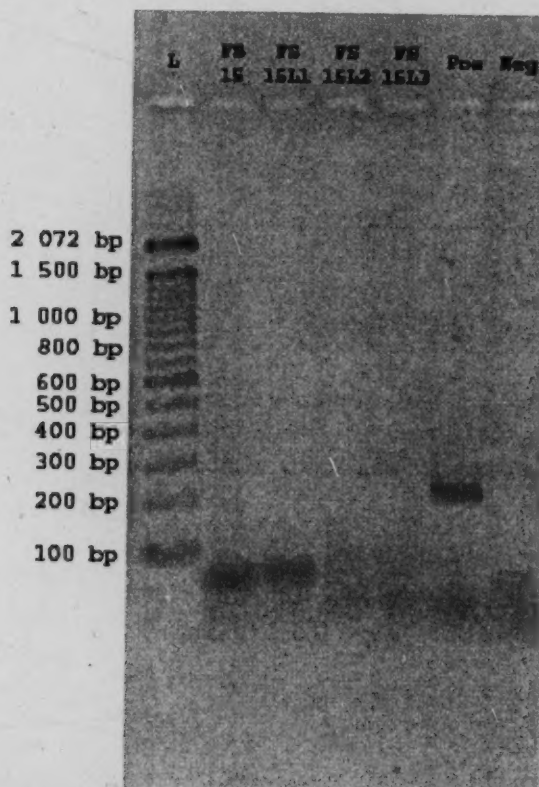


Figure 13: Visualization of PCR products on an agarose gel for detection of DNA contamination in total RNA isolated from wild type *Listeria monocytogenes* FS 15 and leucocin-resistant variants FS 15L1, FS 15L2, and FS 15L3 after treatment with DNA-free™

L = 100 bp ladder, Pos = *Listeria innocua* ATCC 33090 DNA, Neg = negative control

4.6 Restriction fragment differential display analysis of total RNA

After performing PCR to amplify the cDNA, and visualization on a sequencing gel, 6 of the 32 reactions gave profiles of poor quality that either contained too few bands or did not have clear bands. Several attempts to correct the problem, including running new PCR and loading new gels, did not yield improved results. However, the other 26 reactions did result in bands that could be analyzed. Visually, a total of nine bands were selected for densitometry. Four bands from the *leu^R* variants had a significantly higher ($P < 0.05$) signal intensity compared with the wild type strain. These bands were extracted from the gel and named fragments JM1 to JM4. Figure 14 is a representative profile that contains a potential differentially expressed gene fragment. The profile contains fragment JM3.

4.6.1 Cloning of differentially expressed fragments

Fragments JM1 to JM4 were cloned into plasmid pCR[®]2.1 to create plasmids pJM1 to pJM4. The plasmids were required as a source of template so each fragment could be further analyzed by dot blot analysis. *Escherichia coli* TOP10 cells were transformed with each plasmid and selected as blue colonies on LB agar containing Km and X-Gal. The resulting strains were named *E. coli* JM1, *E. coli* JM2, *E. coli* JM3, and *E. coli* JM4.

4.6.2 Plasmid isolation and amplification of inserts

Plasmids pJM1 to pJM4 were isolated from *E. coli* strains JM1 to JM4 using miniprep preparation by alkaline lysis with SDS. The plasmids served as template for PCR amplification of the cloned fragments. The reaction products were electrophoresed on a 1.5% agarose gel, which is shown in Figure 15. A standard curve was constructed from Figure 15, using the migration distances of each band in the 100 bp ladder and the pCR[®]2.1 amplicon, to calculate the sizes of each reaction product from

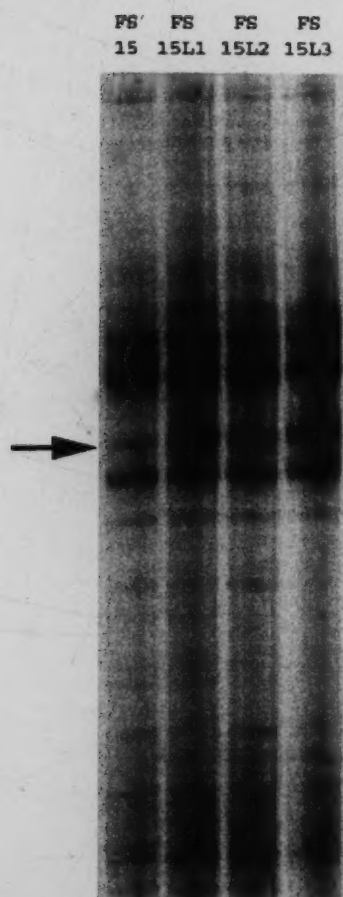


Figure 14: Autoradiogram of an RFDD profile of total RNA isolated from wild type *L. monocytogenes* FS 15 and leucocin-resistant variants FS 15L1, FS 15L2, and FS 15L3 showing a potential differentially displayed gene fragment denoted by the arrow

plasmids pJM1 to pJM4. The calculated sizes were 296, 343, 311, and 351 bp for the products of pJM1 to pJM4, respectively. The size of the pCR[®]2.1 amplicon is known to be 176 bp, and this consists of the 89 bp and 87 bp sequences that flank the insert site of pCR[®]2.1. Subtraction of the 176 basepairs of vector DNA yielded insert sizes of 120, 167, 135, and 175 bp. These were the actual sizes of fragments JM1, JM2, JM3, and JM4, respectively.

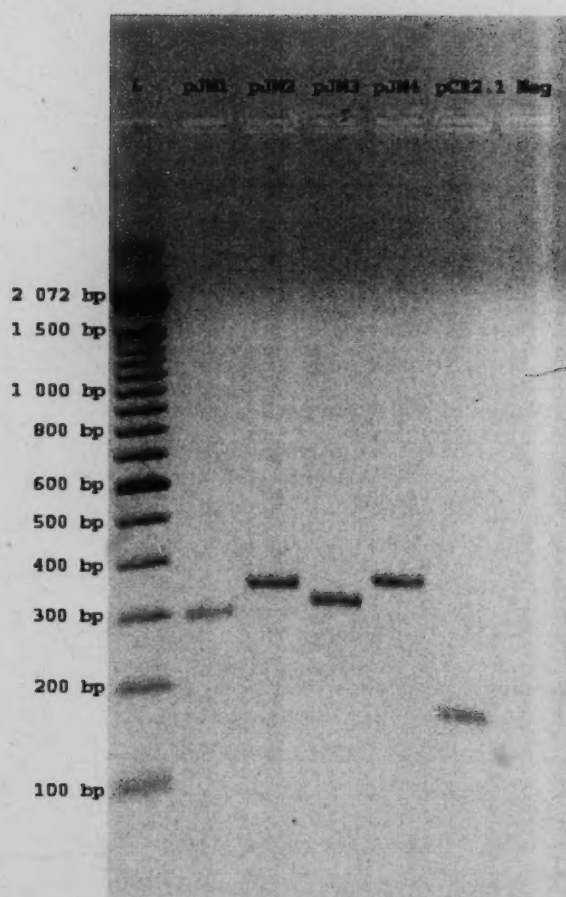


Figure 15: Agarose gel of PCR amplification of cloned differentially expressed fragments from plasmids pJM1, pJM2, pJM3, and pJM4 for determination of insert size

L = 100 bp ladder, pCR2.1 = vector pCR[®]2.1 with no insert, Neg = negative control

4.6.3 Dot blot confirmation of differential expression

Dot blots were performed to confirm the results of RFDD analysis. Fragments JM1 to JM4 were prepared by PCR from plasmids pJM1 to pJM4, radiolabeled, and used to probe membranes spotted with denatured total RNA from *L. monocytogenes* FS 15, FS 15L1, FS 15L2, and FS 15L3. Duplicate membranes were also probed with radiolabeled Eub 338, which binds to 16S rRNA, to accurately determine the amount of RNA in each spot. The expression of each gene fragment was reported as a ratio of the signal intensity

of the spot when probed with an unknown fragment, and the signal intensity of the spot when probed with Eub 338.

An example of a dot blot with probe JM4 is shown in Figure 16. The probe hybridized with RNA from *L. monocytogenes* and not with RNA from *E. coli*. The calculated intensity ratios of probes JM2, JM3, and JM4 were significantly lower ($P < 0.05$) for wild type *L. monocytogenes* FS 15 compared with the three *leu*^R variants. However, the increases in gene expression for the *leu*^R variants were less than two-fold for each of the three fragments. The blot with probe JM1 did not have any signal, and subsequent blots with freshly-prepared membranes and probes did not yield any signal, even with less stringent washing conditions.

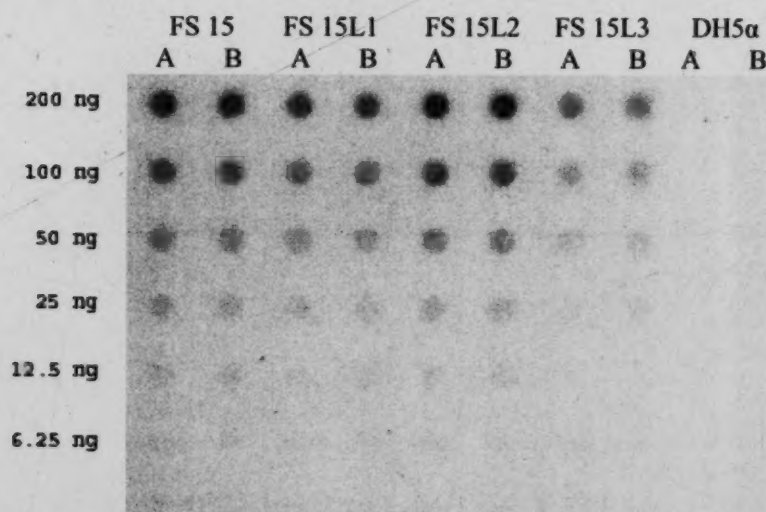


Figure 16: Autoradiogram of dot blot hybridization of fragment JM4 with total RNA from *E. coli* DH5α, wild *L. monocytogenes* FS 15, and *leu*^R variants FS 15L1, FS 15L2, and FS 15L3 (Amount of RNA is shown on left)

4.7 Sequencing of differentially expressed fragments

The unknown fragments were sequenced so their identity could be determined from databases using nucleotide sequence homology searches. Sequencing was performed on all fragments, despite the inability to confirm the differential expression of fragment JM1.

The sequences and sizes were obtained for each cloned fragment. The sizes in basepairs are 119, 167, 139, and 175 for fragments JM1 to JM4, respectively. Using the basic local alignment search tool (BLAST), searches of the NCBI databases identified JM2, JM3, and JM4 as fragments of 23S ribosomal DNA from *L. monocytogenes*. Fragment JM1 was identified as part of gene *lmo0196*, which encodes a product similar to the SpoVG protein of *Bacillus subtilis*. The sequence of each gene fragment and results of the homology searches can be found in the appendix.

5 Discussion and Conclusions

In this study, *leu^R* variants of *L. monocytogenes* were generated from exposure to partially purified leucocin A. In spot-on-lawn assays, they were able to grow confluent lawns in the presence of 6 400 AU/mL. In deferred inhibition assays, they were also able to grow on agar that contained a 16 h culture of the leucocin producer *Leuconostoc gelidum* UAL187. In contrast, the wild type strains could not grow in the presence of > 100 AU/mL and zones of inhibition were observed in deferred inhibition assays. Thus the *leu^R* variants were at least 64 times more resistant to leucocin A than the wild type strains. The IC_{50} values of the wild type and *leu^R* variants used in this study were not determined, thus conclusions as to whether the resistant variants are intermediately- or highly-resistant to leucocin cannot be made. Sensitive strains have IC_{50} values 2.4 to 4 times lower than intermediately-resistant strains, and 500 times lower than highly-resistant strains (Vadyvaloo *et al.* 2002). The level of bacteriocin resistance is relevant to gene expression as demonstrated in a study by Vadyvaloo *et al.* (2004a) that determined that in comparison with the wild type strains, expression of *mptA* was greatly decreased (1 000-fold) for highly-resistant strains but only moderately decreased (fourfold) for intermediately-resistant strains. Thus it is reasonable to assume that if other genes are differentially expressed in *leu^R* *L. monocytogenes*, the amount of differential expression could also vary depending on the level of leucocin resistance.

Analysis of wild type and *leu^R* *L. monocytogenes* by RAPD confirmed that the *leu^R* variants generated in this study were descended from the corresponding wild type strains. Each wild type strain had a RAPD profile similar to the profiles obtained for its *leu^R* variants. Analysis by RAPD also differentiated among strains, since the wild type *L. monocytogenes* ATCC 15313, CDC 7762, and FS 15 all had different profiles. Some studies have shown that use of a single primer for RAPD characterization can result in identical profiles for strains from different serotypes (Lawrence *et al.* 1993, Farber and Addison 1994). As a result, many investigators routinely use two or more different primers in RAPD analysis for typing purposes (Farber and Addison 1994, Boerlin *et al.* 1995, Hof *et al.* 2000). In this study, profiles that were similar using primer OPM-01 were also similar when primer M13 was used. Additionally, the RAPD profiles of *L.*

monocytogenes ATCC 15313, CDC 7762, and FS 15 were distinctly different from each other.

D-values are a useful means for quantitating stress tolerance. In this study, it was difficult to visually identify differences among survival curves, thus the use of D-values facilitated statistical analysis to determine differences among survival curves.

Comparison of the D-values for the wild type strains indicated that there was no significant difference ($P > 0.05$) among them for either heat or acid tolerance. *L. monocytogenes* FS 15 was more heat tolerant than the other strains at 60°C; however, that distinction was not apparent when the wild type strains were heat-shocked prior to exposure to lethal heat at 60°C.

Previous studies by other investigators have suggested that bacteriocin-resistant strains of *L. monocytogenes* have increased tolerance to environmental stresses such as high temperature, low pH, and high salt concentrations (Gao 2001, Aguilar 2004). In this study, based on statistical analysis of the D-values, the heat tolerance of *leu*^R variants of *L. monocytogenes* ATCC 15313 and CDC 7762 was the same as that of the wild type strains. Only one *leu*^R variant of *L. monocytogenes* FS 15 had a D-value that was significantly different from the wild type strain. This different phenotype could be due to a change in gene expression that was responsible for leucocin resistance. Studies on the basis of resistance to class IIa bacteriocins have shown that although it results directly from inactivation of the σ^{54} -dependent *mpt* operon, which encodes the subunits of a mannose PTS permease, it can also arise from inactivation of *rpoN* or *manR*, which encode σ^{54} and its activator (Robichon *et al.* 1997, Dalet *et al.* 2001). Inactivation of *rpoN* or *manR* would be expected to have more widespread effects on the expression of other genes in the cell compared with inactivation of just the structural genes of the mannose PTS (Gravesen *et al.* 2002b). There is currently no evidence that σ^{54} is involved in the stress responses of *L. monocytogenes*, but that does not preclude the involvement of stress response genes in the mechanism of resistance of *L. monocytogenes* to class IIa bacteriocins.

A few researchers have investigated the effect of bacteriocin resistance on the ability of strains to tolerate environmental stress (Modi *et al.* 2000, Gravesen *et al.* 2002a, Aguilar 2004). Modi *et al.* (2000) determined that nisin-resistant strains of *L.*

monocytogenes Scott A had the same D-values as the wild type strain at 55, 60, and 65°C, and concluded that heat tolerance was not affected by resistance to this Type A lantibiotic. In a study on the resistance of *L. monocytogenes* to the class IIb bacteriocin brochocin-C, Gao (2001) identified seven differentially expressed genes in resistant strains, six of which were overexpressed in heat- and acid-shocked cells. Although the strains themselves were not tested to determine their heat and acid tolerance in comparison to the wild type strain, the author concluded that resistance to brochocin-C causes altered gene expression that resembles changes in gene expression in response to heat and acid stress. Aguilar (2004) studied the thermotolerance of *L. monocytogenes* Scott A and ATCC 15313 that were resistant to brochocin-C and leucocin A and found that heat tolerance was higher in the leu^R strain of *L. monocytogenes* ATCC 15313 as compared with the wild type strain, but no difference was found in the brochocin-resistant strain. Aguilar (2004) found that *L. monocytogenes* Scott A strains had the same tolerance to heat, regardless of bacteriocin-susceptibility, which demonstrates that the phenomenon of increased thermotolerance as a result of bacteriocin resistance may not occur in all strains of *L. monocytogenes*. Wild type and leu^R *L. monocytogenes* ATCC 15313 were studied in this investigation, but in contrast to the results of Aguilar (2004), no difference was found in their D-values determined at 60°C. However, unlike Aguilar's (2004) study, cultures were heat-shocked at 48°C for 20 min prior to exposure to the lethal temperature of 60°C. It is known that exposure to sublethal temperatures increases the ability of *L. monocytogenes* to tolerate lethal temperatures (McMahon *et al.* 2000, Lin and Chou 2004).

In a broader context, the results of this study suggest that resistance to leucocin A may increase the thermotolerance of *L. monocytogenes*, but not for all strains, and not for all variants of the same strain as determined by RAPD analysis. The strains used in this study all belong to different serotypes; *L. monocytogenes* ATCC 15313, CDC 7762, and FS 15 are serotypes 1/2a, 4b (Anonymous 1998), and 1/2b respectively. There is evidence that the ability of *L. monocytogenes* to survive treatment with lethal heat may be linked with serotype, with serotypes 4c, 4d, and 3c being less thermotolerant than serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 4a, and 4b (Francis and O'Beirne 2005). In a study on the alternative sigma factor σ^B , Moorhead and Dykes (2003) used *sigB* null mutants to

determine that σ^B is important for tolerance to osmotic stress, low pH, and high temperature in a serotype 1/2a strain of *L. monocytogenes*, but it has little or no effect in a strain belonging to serotype 4c. Thus it is not unexpected that only certain serotypes are affected by changes in the expression of a specific gene, and if leucocin resistance resulted in upregulation of such a gene, it could increase heat tolerance in strains from certain serotypes and not others. So far, analysis of mRNA and protein expression in *L. monocytogenes* resistant to class IIa bacteriocins has not detected altered expression of stress-related genes (Gravesen *et al.* 2000, Ramnath *et al.* 2000). Further work with a wider variety of *L. monocytogenes* strains from each serotype, and a larger number of *leu*^R variants of each strain may help determine how widespread this phenomenon is, and which strains and serotypes are affected. There exists the possibility that this phenotype may be the result of a point mutation that is unrelated to the mutation that resulted in leucocin-resistance, and this development also needs to be investigated.

The possibility of increased thermotolerance of bacteriocin resistant *L. monocytogenes* raises concerns for the food industry. If *L. monocytogenes* was exposed to a class IIa bacteriocin such as leucocin or pediocin in a food product, and the strain was able to develop bacteriocin resistance, it could also gain increased heat tolerance as a result. In a food that is normally pasteurized to kill contaminating bacteria, there is potential that the heat tolerant bacteria would be able to survive processing, whereas that opportunity would not arise in the absence of bacteriocin treatment.

Determination of the acid tolerance of wild type and *leu*^R *L. monocytogenes*, showed that the *leu*^R variants of *L. monocytogenes* ATCC 15313 and CDC 7762 were less acid tolerant than the wild types. The D-values of *leu*^R variants were significantly less than that of the wild type strains, indicating that they were killed faster by 0.1 M acetic acid at a pH of 3.6. For wild type and *leu*^R *L. monocytogenes* FS 15, there was no difference in acid tolerance, but as with the heat tolerance assays there was a fairly large amount of variability in the data.

Aguilar (2004), found that a brochocin-resistant strain of *L. monocytogenes* ScottA was more acid-tolerant than wild type or *leu*^R strains. Unlike the results of the current study, Aguilar (2004) found that a *leu*^R strain of *L. monocytogenes* ATCC 15313 was more acid-tolerant than the wild type or brochocin-resistant strains. The difference

in the results between the two experiments cannot be easily accounted for, since Aguilar (2004) used techniques similar to those in this study for determination of acid tolerance.

In a study of pediocin-resistant and nisin-resistant strains of *L. monocytogenes*, Gravesen *et al.* (2002a) determined that resistance to either pediocin or nisin did not result in a change in acid tolerance, as represented by the growth rate. However, this study examined growth in the presence of brain heart infusion broth (BHI) acidified to a pH of 6.25. The higher pH would result in less acid stress being placed on the organisms, and therefore any changes in the sensitivity of the bacteriocin-resistant cells to acid might not be noticeable. McEntire *et al.* (2004) discovered that a nisin-resistant mutant of *L. monocytogenes* ScottA was more sensitive to acid than the wild type when it was grown in BHI acidified to pH 3.0 with lactic acid. The authors determined that this phenotype was due to increased activity of ATPase in the nisin-resistant mutant, which caused a depletion of intracellular ATP and energy, resulting in growth inhibition. However, the involvement of ATPase in nisin resistance has not been described before, so this strain should be studied in more depth.

In this study, the conclusions reached must be viewed in the context of other mechanisms of acid tolerance, such as modified fatty acid content (van Schaik *et al.* 1999). In the current study, tolerance to acidic conditions was determined in a solution of acetic acid. Thus there was neither a source of glutamate for the glutamate decarboxylase system to function (Cotter *et al.* 2001a), nor an energy source to provide the energy required for ATPase activity (Cotter *et al.* 2000). For a broader view of acid tolerance, experimental conditions could include an acidified growth medium containing glutamate and an energy source.

Gravesen *et al.* (2004) found that a *leu*^R strain of *L. monocytogenes* had less than half the level of expression of the genes *dat* and *dltA* compared with the wild type strain. The gene *dltA* is required for D-alanine esterification of lipoteichoic acids and results in an increase in the positive charge of the cell wall (Delcour *et al.* 1999, Vadyvaloo *et al.* 2004a). Boyd *et al.* (2000) discovered that a deletion mutation in the *dlt* operon resulted in increased acid sensitivity in *Streptococcus mutans*, and this was due to increased permeability of the membrane to protons. The authors postulated that the increased susceptibility to acid was the result of energy expenditure to maintain a proton gradient,

because the glucose in the medium was depleted, but the final population was only two-thirds that of the wild type population. The increased permeability to protons is thought to place an increased demand on the F_0F_1 -ATPase to pump protons out of the cell, and the ATP used up in this process depletes the pool of energy available for growth and synthesis of proteins involved in the acid tolerance response (Boyd *et al.* 2000). Boyd *et al.* (2000) do not rule out the possibility that if ATPase activity is not able to keep up with maintenance of intracellular pH, acidification of the cytoplasm could also contribute to cell death.

In their study on acid-adapted *L. monocytogenes* ScottA, van Schaik *et al.* (1999) determined that the membranes of acid-adapted cells had a significant ($P < 0.05$) increase in saturated straight-chain fatty acids compared with unadapted cells. Compared with wild type strains, *leu*^R *L. monocytogenes* have a higher content of unsaturated fatty acids (Vadyvaloo *et al.* 2002). These findings suggest that an increase in the saturated fatty acid content of the cell membrane results in increased acid tolerance. It is possible that increased levels of unsaturated fatty acids would decrease acid tolerance, but Quivey *et al.* (2000) have shown otherwise. Acid-adapted *S. mutans* had an increased ratio of unsaturated ($C_{18:1}$ and $C_{20:1}$) to saturated ($C_{14:0}$ and $C_{16:0}$) fatty acids in the membrane compared with cells which were not acid-adapted (1.20 compared with 0.33, respectively). It is clear that further study is required to determine the effect of changes in fatty acid content on the tolerance of cells to low pH.

The findings of this study show that some strains of *L. monocytogenes* can become more acid sensitive when they acquire bacteriocin resistance. In a food system where a class IIa bacteriocin such as leucocin A is used as a biopreservative, bacteriocin resistance would further decrease the ability of the bacteria to survive since they would be more susceptible to low pH conditions.

The salt tolerance of *L. monocytogenes* was assayed by examining the survival of wild type and *leu*^R variants in a phosphate buffer containing 8% NaCl. Most of the wild type strains and *leu*^R variants tested displayed a decrease of up to two logs in cell population that lasted up to 18 days before stabilizing for the remainder of the experiment. However, the population of the wild type strain of *L. monocytogenes* CDC 7762 increased over the first six days of the experiment. In all cases, the population

stabilized after 18 days of incubation. Stabilization of the cell population could be due to bacteriostatic effects from the sodium chloride, as well as a stationary phase-like response due to the lack of nutrients in the phosphate buffer.

Although there were slight differences in the survivor curves during the initial six hours of the experiment, the results of this study indicate that there is little difference between the salt tolerance of the wild type strains and their *leu*^R variants.

Aguilar (2004) examined the survival of wild type, brochochin-, and leucocin-resistant *L. monocytogenes* ScottA and ATCC 15313 in 20% NaCl. He determined that the salt tolerance of almost all strains tested were the same, with only the *leu*^R *L. monocytogenes* ATCC 15313 strain displaying increased salt tolerance. As in the current study, Gravesen *et al.* (2002a) were not able to detect any difference in sensitivity of pediocin- or nisin-resistant *L. monocytogenes* to 6.5% NaCl as compared with wild type strains.

Most studies that focus on determining salt tolerance in *L. monocytogenes* use either growth rate or time until growth is detected as the determinants of halotolerance (McClure *et al.* 1989, Cole *et al.* 1990, Sleator *et al.* 2000, Gravesen *et al.* 2002a). This complicates comparison of the current results with published work, since the purpose of this study was to determine the survival of a large number of cells in a saline environment, rather than the growth of a small number of cells.

In a study on the tolerance of *L. monocytogenes* to high salt conditions, McClure *et al.* (1989) determined that *L. monocytogenes* was capable of growth in 10% NaCl at 25°C and neutral pH. Cole *et al.* (1990) later reported that *L. monocytogenes* can grow in up to 12% NaCl at 30°C and neutral pH, but 38.3 days of incubation were required to detect growth. Furthermore, 14% NaCl did not permit cell growth within 60 days. Sleator *et al.* (2000) determined that the specific growth rate of wild type *L. monocytogenes* LO28 was almost zero when cells were grown in medium containing 10% NaCl. However, when halotolerance was determined from survival of cells rather than growth, the conclusion was that *L. monocytogenes* is tolerant of salt concentrations much higher than 10%. In a recent study, Liu *et al.* (2005) studied the ability of *L. monocytogenes* to survive in varying concentrations of NaCl, and they concluded that the bacterium could survive in a saturated salt solution of 40% NaCl after 20 h. However,

the investigators did not plot survival curves of *L. monocytogenes* and determine D-values. Instead, they enumerated samples at the end of each experiment (20 h for the saturated solution, 1 h for all other concentrations) and compared the final population to the initial population. Limited conclusions can be drawn from their study due to the short duration of the exposure to salt and the absence of survival data over a time course.

With the knowledge that salt tolerance in *L. monocytogenes* depends heavily on uptake of the compatible solutes glycine betaine and carnitine (Hill *et al.* 2002), it is apparent that a more suitable assay of halotolerance could have involved a growth medium containing NaCl, glycine betaine, carnitine, and an energy source for their uptake. In this study, endogenous proline synthesis would have been the only identifiable source of tolerance to the hypertonic salt conditions, and synthesis would have been limited due to the lack of a carbon source in the phosphate buffer. The use of phosphate buffer instead of a growth medium may account for some of the variability in the results, but that is only speculation at this point.

In this study, strains of *L. monocytogenes* that had a decrease in acid tolerance (ATCC 15313 and CDC 7762) with bacteriocin resistance did not have a change in heat tolerance. Likewise, the strain that had increased heat tolerance (FS 15) had the same acid tolerance for its leu^R variants. The implications for this pattern are unknown, but it suggests that the mechanism behind the decrease in acid tolerance is unrelated to the cause of increased heat tolerance.

To determine which genes were responsible for the changes in stress-tolerance of the leu^R variants, restriction fragment differential display was used to screen for genes that had higher or lower expression compared with the wild type strain. Since the screening process is relatively expensive, RFDD-PCR was only performed on one wild type strain and its three leu^R variants. *L. monocytogenes* FS 15 was chosen for analysis primarily because of the increased thermotolerance of one of its leu^R variants.

Analysis of gene expression by RFDD-PCR yielded four fragments of potential differentially expressed genes. Confirmation of these results by dot blot analysis revealed that three of the fragments (JM2, JM3, and JM4) had higher expression in total RNA isolated from the leu^R variants of *L. monocytogenes* FS 15 than the wild type strain. However, differential expression of the fourth fragment (JM1) could not be verified from

dot blots. The stringency of the washes for the dot blots was lowered, but still resulted in no hybridization signal. It was possible that the expression level of the complementary RNA for fragment JM1 might be too low for detection by dot blot. Northern blots, which are similar to dot blots, also suffer from a detection limit that may be too low for rarely-expressed mRNA species, so more sensitive techniques may be required (Medhurst *et al.* 2000, González *et al.* 2003). Quantitative reverse transcriptase-PCR (RT-PCR) coupled with TaqMan[®] real time PCR (Applied Biosystems) would be a more sensitive approach, and may have allowed for confirmation of differential expression of fragment JM1.

The three fragments (JM2, JM3, and JM4) that had significantly higher expression in the *leu^R* variants turned out to be fragments of the 23S rRNA gene. This was unexpected since rRNA is generally not considered to be differentially expressed; in fact, it is used in studies as an internal control to ensure equal amounts of RNA or cells are being examined (Bubert *et al.* 1999, Bibiloni *et al.* 2005). This raised the question of whether a difference in signal intensity necessarily translates into a phenotypic difference in gene expression. In this study, there were two steps where differences were determined. The first instance was during RFDD-PCR analysis, and the second was during confirmation of differential expression using dot blots. There is currently no standard difference in expression level that researchers use to identify differential bands in differential display PCR. Some studies have relied on visual identification of differential bands (Fleming *et al.* 1998), while others used densitometry or fluorescence analysis to select bands with greater than five-fold differences in signal intensity (Gravesen *et al.* 2000, Gao 2001). However, it has been observed that differential bands with similar intensities in a differential display profile can have very different expression levels when tested with independent techniques such as TaqMan[®] quantitative PCR (Medhurst *et al.* 2000). The detection of false-positives resulting from differential display has been documented in both prokaryotic and eukaryotic systems (Liang *et al.* 1993, Fleming *et al.* 1998, Medhurst *et al.* 2000); therefore, the crucial determination of expression level is during the confirmation step. In this study, the confirmation step was dot blot analysis, and although a statistically significant difference in gene expression was determined for fragments JM2, JM3, and JM4, they amounted to less than two-fold increases compared with the wild type strain. Considering that the *mptA* gene of *L.*

monocytogenes is expressed in leucocin- and pediocin-resistant strains at levels 1 000-fold lower than levels in wild type strains (Vadyvaloo *et al.* 2004a), it is apparent that the fragments of the 23S rRNA gene, which had less than a two-fold increase in expression, were not differentially expressed in leu^R *L. monocytogenes* used in the current study.

It is unclear whether or not *spoVG*, which was identified by fragment JM1, is involved in leucocin resistance in *L. monocytogenes* because differential expression of the gene could not be verified. Moreover, although a gene similar to *spoVG* is present in the genome of *L. monocytogenes* EGD-e (see Appendix), there are no published studies on its function in this non-spore-forming organism. In *Bacillus* spp., expression of *spoVG* is repressed by the global gene repressor AbrB and stimulated by the alternative sigma factor σ^H (Carter and Moran 1986, Robertson *et al.* 1989). The SpoVG protein prevents formation of the asymmetric septum, which occurs during the early stages of sporulation in *Bacillus* spp.; thus it may be responsible for regulating the timing of the sporulation process (Matsuno and Sonenshein 1999). Its expression is also induced upon mild heat shock, suggesting that SpoVG has the additional function of preventing sporulation during exposure to mild environmental stress (Periago *et al.* 2002).

A noteworthy result of this study is the absence of differential expression of any of the genes that have been identified by other researchers as differentially expressed in strains of *L. monocytogenes* that are resistant to class IIa bacteriocins. These genes include a phospho- β -glucosidase and a β -glucoside-specific PTS enzyme II permease identified by RFDD-PCR (Gravesen *et al.* 2000), and a mannose-specific PTS enzyme IIAB subunit identified by 2D-gel electrophoresis and quantitative real-time PCR (Ramnath *et al.* 2000, Gravesen *et al.* 2002b, Vadyvaloo *et al.* 2004a). Because 6 of the 32 reactions could not be analyzed, one can only speculate that if any of these genes were differentially expressed in the leu^R variants in this study, then the bands may have been present in those profiles.

Restriction fragment differential display PCR is a useful screening method for examining differential gene expression. Previous researchers have established the advantages of the technique as well as differential display in general (Liang *et al.* 1993, Gravesen *et al.* 2000, Gao 2001). It can be used to screen for all genes that are differentially expressed under a given set of conditions, which is useful when little or no

sequence information is available for the organism in question, or no particular genes of interest are being searched for. The procedure generates discrete bands that can easily be excised from the gel for further study, and different RNA samples can be easily compared in adjacent lanes on the same gel. The technique is also relatively simple when a commercial kit (Qbiogene) is used and it can be performed with common and inexpensive laboratory equipment.

There is, however, one major drawback of differential display PCR. In this study, three of the four differential bands chosen for confirmation by dot blot turned out to be false-positives. A high incidence of false-positives from differential display PCR has been reported previously (Liang *et al.* 1993, Fleming *et al.* 1998), but it is unclear how prevalent the problem is with the restriction fragment differential display PCR technique used in this study, since others who have used it did not report concerns about false-positives (Gravesen *et al.* 2000, Gao 2001). False-positives are thought to arise primarily from the presence of multiple fragments in a band (Debouck 1995). In this study, the three confirmed false-positives arose from ribosomal RNA. Prokaryotic mRNA is difficult to separate from total RNA, unlike eukaryotic mRNA which can be purified or preferentially amplified by virtue of its 3' poly(A) tail. It is evident that removal of rRNA from the sample prior to analysis would decrease the number of bands generated and the incidence of false-positives, thereby improving the starting material for RFDD-PCR analysis.

Few methods are available for removal of rRNA from total prokaryotic RNA. Amara and Vijaya (1997) used yeast poly(A) polymerase under conditions that allowed them to polyadenylate *E. coli* mRNA but not 16S and 23S rRNA. The mRNA was then bound to oligo(dT) beads, separated from unbound RNA by centrifugation, and purified by elution from the beads. Another approach is employed by Ambion's MICROBExpress™ Bacterial mRNA Enrichment Kit. This procedure involves the use of oligonucleotides linked to magnetic beads. The oligonucleotides bind to 16S and 23S rRNA, and after hybridization with a total RNA sample, the rRNA-bound beads are immobilized on the side of the tube with a magnet while the remaining RNA is transferred to a new tube. Small RNA molecules such as 5S rRNA and tRNA can be

removed from the sample using Ambion's MEGAclean™ Kit, yielding a sample containing only mRNA.

From the results of this study, it is clear that more work is needed to examine the effect of leucocin resistance on stress tolerance. In terms of heat and acid tolerance, there appear to be strain-specific and potentially serovar-specific factors involved, but no clear patterns were identified. For heat tolerance, one leu^R variant of *L. monocytogenes* strain FS 15 had a higher thermotolerance than the wild type, and there was no difference in the heat tolerance of the other strains. For acid tolerance, the leu^R variants of *L. monocytogenes* ATCC 15313 and CDC 7762 were less tolerant than the wild type strains, while there was no difference between wild type and leu^R FS 15. Leucocin resistance appears to have no effect on salt tolerance. Thus it appears that for some strains, resistance to class IIa bacteriocins can make *L. monocytogenes* more tolerant to heat, raising concerns about the ability of such strains to survive pasteurization and other heat treatments used for food. On the other hand, some strains become more susceptible to low pH, which would decrease their ability to survive in acidic foods.

Further work is required to establish the genetic determinants that are involved in the altered stress tolerance responses of class IIa bacteriocin-resistant *L. monocytogenes*. In this study, RFDD-PCR did not identify any differentially expressed genes in leu^R bacteria. However, a modified procedure using purified bacterial mRNA as starting material could yield better results by eliminating bands produced by other RNA species.

6 References

- Abranches, J., Y. Y. Chen and R. A. Burne (2003). Characterization of *Streptococcus mutans* strains deficient in EIIAB Man of the sugar phosphotransferase system. *Appl Environ Microbiol* **69**: 4760-9.
- Aguilar, J. (2004). Response of bacteriocin resistant *Listeria monocytogenes* strains to preservation factors. Department of Agricultural, Food, and Nutritional Science. Edmonton, University of Alberta: 89.
- Ahn, C. and M. E. Stiles (1990). Plasmid-associated bacteriocin production by a strain of *Carnobacterium piscicola* from meat. *Appl Environ Microbiol* **56**: 2503-10.
- Allison, G. E., C. Fremaux and T. R. Klaenhammer (1994). Expansion of bacteriocin activity and host range upon complementation of two peptides encoded within the lactacin F operon. *J Bacteriol* **176**: 2235-41.
- Amann, R. L., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux and D. A. Stahl (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919-25.
- Amara, R. R. and S. Vijaya (1997). Specific polyadenylation and purification of total messenger RNA from *Escherichia coli*. *Nucleic Acids Res* **25**: 3465-70.
- Anonymous (1998). Multistate outbreak of listeriosis -- United States, 1998. *Morb Mortal Wkly Rep* **47**: 1085-1086.
- Anonymous (2002). Outbreak of listeriosis--northeastern United States, 2002. *MMWR Morb Mortal Wkly Rep* **51**: 950-1.
- Ariazi, E. A. and M. N. Gould (1996). Identifying differential gene expression in monoterpene-treated mammary carcinomas using subtractive display. *J Biol Chem* **271**: 29286-94.
- Aureli, P., G. C. Fiorucci, D. Caroli, G. Marchiaro, O. Novara, L. Leone and S. Salmaso (2000). An outbreak of febrile gastroenteritis associated with corn contaminated by *Listeria monocytogenes*. *N Engl J Med* **342**: 1236-41.
- Bayles, D. O. (2003). Changes in heat resistance resulting from pH and nutritional shifts of acid-adapted and non-acid-adapted *Listeria monocytogenes* Scott A. *J Food Prot* **67**: 316-321.
- Becker, L. A., M. S. Cetin, R. W. Hutkins and A. K. Benson (1998). Identification of the gene encoding the alternative sigma factor sigmaB from *Listeria monocytogenes* and its role in osmotolerance. *J Bacteriol* **180**: 4547-54.

- Beumer, R. R., M. C. te Giffel, E. Spoorenberg and F. M. Rombouts (1996). *Listeria* species in domestic environments. *Epidemiol Infect* **117**: 437-42.
- Bibiloni, R., M. A. Simon, C. Albright, B. Sartor and G. W. Tannock (2005). Analysis of the large bowel microbiota of colitic mice using PCR/DGGE. *Lett Appl Microbiol* **41**: 45-51.
- Bierbaum, G. and H. G. Sahl (1987). Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of N-acetylmuramoyl-L-alanine amidase. *J Bacteriol* **169**: 5452-8.
- Blendon, D. C., E. H. Kampelmacher and M. J. Torres-Anjel (1987). Listeriosis. *J Am Vet Med Assoc* **191**: 1546-51.
- Boerlin, P., E. Bannerman, F. Ischer, J. Rocourt and J. Bille (1995). Typing *Listeria monocytogenes*: a comparison of random amplification of polymorphic DNA with 5 other methods. *Res Microbiol* **146**: 35-49.
- Bortolussi, R. and W. F. Schlech, 3rd (1995). Listeriosis. Infectious diseases of the fetus and newborn infant. J. S. Remington and J. O. Klein. Philadelphia, W.B. Saunders Company: 1055-1073.
- Boyd, D. A., D. G. Cvitkovitch, A. S. Bleiweis, M. Y. Kiriukhin, D. V. Debabov, F. C. Neuhaus and I. R. Hamilton (2000). Defects in D-alanyl-lipoteichoic acid synthesis in *Streptococcus mutans* results in acid sensitivity. *J Bacteriol* **182**: 6055-65.
- Breukink, E., I. Wiedemann, C. van Kraaij, O. P. Kuipers, H. Sahl and B. de Kruijff (1999). Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. *Science* **286**: 2361-4.
- Brötz, H., G. Bierbaum, K. Leopold, P. E. Reynolds and H. G. Sahl (1998). The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. *Antimicrob Agents Chemother* **42**: 154-60.
- Bubert, A., Z. Sokolovic, S. K. Chun, L. Papatheodorou, A. Simm and W. Goebel (1999). Differential expression of *Listeria monocytogenes* virulence genes in mammalian host cells. *Mol Gen Genet* **261**: 323-36.
- Cain, D. B. and V. L. McCann (1986). An unusual case of cutaneous listeriosis. *J Clin Microbiol* **23**: 976-7.
- Camilli, A., L. G. Tilney and D. A. Portnoy (1993). Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. *Mol Microbiol* **8**: 143-157.

- Carter, H. L., 3rd and C. P. Moran, Jr. (1986). New RNA polymerase sigma factor under *spo0* control in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **83**: 9438-42.
- Cetin, M. S., C. Zhang, R. W. Hutkins and A. K. Benson (2004). Regulation of transcription of compatible solute transporters by the general stress sigma factor, sigmaB, in *Listeria monocytogenes*. *J Bacteriol* **186**: 794-802.
- Chen, Y. and T. J. Montville (1995). Efflux of ions and ATP depletion induced by pediocin PA-1 are concomitant with cell death in *Listeria monocytogenes* ScottA. *J Appl Bacteriol* **79**: 684-690.
- Cheroutre-Vialette, M., I. Lebert, M. Hebraud, J. C. Labadie and A. Lebert (1997). Effects of pH or a_w stress on growth of *Listeria monocytogenes*. *Int J Food Microbiol* **42**: 71-77.
- Cintas, L. M., M. P. Casaus, C. Herranz, I. F. Nes and P. E. Hernandez (2001). Review: Bacteriocins of lactic acid bacteria. *Food Sci Tech Int* **7**: 281-305.
- Cleveland, J., T. J. Montville, I. F. Nes and M. L. Chikindas (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *Int J Food Microbiol* **71**: 1-20.
- Cole, M. B., M. V. Jones and C. Holyoak (1990). The effect of pH, salt concentration and temperature on the survival and growth of *Listeria monocytogenes*. *J Appl Bacteriol* **69**: 63-72.
- Cotter, P. D., N. Emerson, C. G. Gahan and C. Hill (1999). Identification and disruption of *lisRK*, a genetic locus encoding a two-component signal transduction system involved in stress tolerance and virulence in *Listeria monocytogenes*. *J Bacteriol* **181**: 6840-3.
- Cotter, P. D., C. G. Gahan and C. Hill (2000). Analysis of the role of the *Listeria monocytogenes* F_0F_1 -ATPase operon in the acid tolerance response. *Int J Food Microbiol* **60**: 137-46.
- Cotter, P. D., C. G. Gahan and C. Hill (2001a). A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Mol Microbiol* **40**: 465-75.
- Cotter, P. D., K. O'Reilly and C. Hill (2001b). Role of the glutamate decarboxylase acid resistance system in the survival of *Listeria monocytogenes* LO28 in low pH foods. *J Food Prot* **64**: 1362-8.
- Crandall, A. D. and T. J. Montville (1998). Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. *Appl Environ Microbiol* **64**: 231-7.

- Dalet, K., C. Briand, Y. Cenatiempo and Y. Héchard (2000). The *rpoN* gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. *Curr Microbiol* **41**: 441-3.
- Dalet, K., Y. Cenatiempo, P. Cossart and Y. Héchard (2001). A sigma(54)-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. *Microbiology* **147**: 3263-9.
- Dalton, C. B., C. C. Austin, J. Sobel, P. S. Hayes, W. F. Bibb, L. M. Graves, B. Swaminathan, M. E. Proctor and P. M. Griffin (1997). An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk. *N Engl J Med* **336**: 100-5.
- Davis, M. J., P. J. Coote and C. P. O'Byrne (1996). Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiology* **142** (Pt 10): 2975-82.
- Debouck, C. (1995). Differential display or differential dismay? *Curr Opin Biotechnol* **6**: 597-599.
- Delcour, J., T. Ferain, M. Deghorain, E. Palumbo and P. Hols (1999). The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek* **76**: 159-84.
- Devlieghere, F., L. Vermeiren and J. Debevere (2004). New preservation technologies: Possibilities and limitations. *Int Dairy J* **14**: 273-285.
- Dincer, A. H. and T. Baysal (2004). Decontamination techniques of pathogen bacteria in meat and poultry. *Crit Rev Microbiol* **30**: 197-204.
- Dramsi, S., I. Biswas, E. Maguin, L. Braun, P. Mastroeni and P. Cossart (1995). Entry of *L. monocytogenes* into hepatocytes requires expression of InlB, a surface protein of the internalin multigene family. *Mol Microbiol* **16**: 251-261.
- Driessen, A. J., H. W. van den Hooven, W. Kuiper, M. van de Kamp, H. G. Sahl, R. N. Konings and W. N. Konings (1995). Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. *Biochemistry* **34**: 1606-14.
- Duguid, J. R., R. G. Rohwer and B. Seed (1988). Isolation of cDNAs of scrapie-modulated RNAs by subtractive hybridization of a cDNA library. *Proc Natl Acad Sci U S A* **85**: 5738-42.
- Dykes, G. A. and J. W. Hastings (1998). Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. *Lett Appl Microbiol* **26**: 5-8.

- Ennahar, S., T. Sashihara, K. Sonomoto and A. Ishizaki (2000). Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol Rev* **24**: 85-106.
- Evans, J. R., A. C. Allen, D. A. Stinson, R. Bortolussi and L. J. Peddle (1985). Perinatal listeriosis: report of an outbreak. *Pediatr Infect Dis* **4**: 237-41.
- Fan, Y. D., J. G. Pastorek, 2nd, F. A. Janney and C. V. Sanders (1989). Listeriosis as an obstetric complication in an immunocompromised patient. *South Med J* **82**: 1044-5.
- Farber, J. M. and C. J. Addison (1994). RAPD typing for distinguishing species and strains in the genus *Listeria*. *J Appl Bacteriol* **77**: 242-50.
- Farber, J. M., E. Daley, F. Coates, N. Beausoleil and J. Fournier (1991). Feeding trials of *Listeria monocytogenes* with a nonhuman primate model. *J Clin Microbiol* **29**: 2606-8.
- Farber, J. M. and P. I. Peterkin (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev* **55**: 476-511.
- Farber, J. M., G. W. Sanders, J. I. Speirs, J. Y. D'Aoust, D. B. Emmons and R. McKellar (1988). Thermal resistance of *Listeria monocytogenes* in inoculated and naturally contaminated raw milk. *Int J Food Microbiol* **7**: 277-86.
- FDA (1991). *Listeria monocytogenes*. Recommendations by The National Advisory Committee on Microbiological Criteria for Foods. *Int J Food Microbiol* **14**: 185-246.
- Ferreira, A., C. P. O'Byrne and K. J. Boor (2001). Role of sigma(B) in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. *Appl Environ Microbiol* **67**: 4454-7.
- Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome and A. L. Reingold (1985). Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *N Engl J Med* **312**: 404-7.
- Fleming, J. T., W. H. Yao and G. S. Sayler (1998). Optimization of differential display of prokaryotic mRNA: application to pure culture and soil microcosms. *Appl Environ Microbiol* **64**: 3698-706.
- Francis, G. A. and D. O'Beirne (2005). Variation among strains of *Listeria monocytogenes*: differences in survival on packaged vegetables and in response to heat and acid conditions. *Food Control* **16**: 687-694.

- Frye, D. M., R. Zweig, J. Sturgeon, M. Tormey, M. LeCavalier, I. Lee, L. Lawani and L. Mascola (2002). An outbreak of febrile gastroenteritis associated with delicatessen meat contaminated with *Listeria monocytogenes*. *Clin Infect Dis* **35**: 943-9.
- Gahan, C. G., B. O'Driscoll and C. Hill (1996). Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. *Appl Environ Microbiol* **62**: 3128-32.
- Gahan, C. G., J. O'Mahony and C. Hill (2001). Characterization of the *groESL* operon in *Listeria monocytogenes*; utilization of two reporter systems (*gfp* and *hly*) for evaluating in vivo expression. *Infect Immun* **69**: 3924-32.
- Gaillard, J. L., P. Berche, C. Frehel, E. Gouin and P. Cossart (1991). Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from Gram-positive cocci. *Cell* **65**: 1127-1141.
- Gao, Y. (2001). Brochocin-C resistance in *Listeria monocytogenes*. Department of Agricultural, Food, and Nutritional Science. Edmonton, University of Alberta: 178.
- Golnazarian, C. A., C. W. Donnelly, S. J. Pintauro and D. B. Howard (1989). Comparison of infectious dose of *Listeria monocytogenes* F5817 as determined for normal versus compromised C57B1/6J mice. *J Food Prot* **52**: 696-701.
- González, T., M. C. Terrón, E. J. Zapico, A. Telléz, S. Yagüe, J. M. Carbajo and A. E. González (2003). Use of multiplex reverse transcription-PCR to study the expression of a laccase gene family in a basidiomycetous fungus. *Appl Environ Microbiol* **69**: 7083-90.
- Graves, L. M., S. B. Hunter, A. R. Ong, D. Schoonmaker-Bopp, K. Hise, L. Kornstein, W. E. DeWitt, P. S. Hayes, E. Dunne, P. Mead and B. Swaminathan (2005). Microbiological aspects of the investigation that traced the 1998 outbreak of listeriosis in the United States to contaminated hot dogs and establishment of molecular subtyping-based surveillance for *Listeria monocytogenes* in the PulseNet network. *J Clin Microbiol* **43**: 2350-5.
- Gravesen, A., A. M. Jydegaard Axelsen, J. Mendes da Silva, T. B. Hansen and S. Knochel (2002a). Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. *Appl Environ Microbiol* **68**: 756-64.
- Gravesen, A., B. Kallipolitis, K. Holmstrom, P. E. Hoiby, M. Ramnath and S. Knochel (2004). *pbp2229*-mediated nisin resistance mechanism in *Listeria monocytogenes* confers cross-protection to class IIa bacteriocins and affects virulence gene expression. *Appl Environ Microbiol* **70**: 1669-79.

- Gravesen, A., M. Ramnath, K. B. Rechinger, N. Andersen, L. Jansch, Y. Héchard, J. W. Hastings and S. Knochel (2002b). High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes*. *Microbiology* **148**: 2361-9.
- Gravesen, A., P. Warthoe, S. Knochel and K. Thirstrup (2000). Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative beta-glucoside-specific PTS system. *Microbiology* **146** (Pt 6): 1381-9.
- Gursky, L. (2004). Bacteriocin production by *Carnobacterium maltaromaticum* UAL26. Department of Agricultural, Food, and Nutritional Science. Edmonton, University of Alberta: 205.
- Harris, L. J., H. P. Fleming and T. R. Klaenhammer (1992). Developments in nisin research. *Food Res Int* **25**: 57-66.
- Harty, J. T., L. L. Lenz and M. J. Bevan (1996). Primary and secondary immune responses to *Listeria monocytogenes*. *Curr Opin Immunol* **8**: 526-30.
- Hasper, H. E., B. de Kruijff and E. Breukink (2004). Assembly and stability of nisin-lipid II pores. *Biochemistry* **43**: 11567-75.
- Hastings, J. W., M. Sailer, K. Johnson, K. L. Roy, J. C. Vederas and M. E. Stiles (1991). Characterization of leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. *J Bacteriol* **173**: 7491-500.
- Hauge, H. H., D. Mantzilas, G. N. Moll, W. N. Konings, A. J. Driessen, V. G. Eijsink and J. Nissen-Meyer (1998). Plantaricin A is an amphiphilic alpha-helical bacteriocin-like pheromone which exerts antimicrobial and pheromone activities through different mechanisms. *Biochemistry* **37**: 16026-32.
- Héchard, Y., C. Pelletier, Y. Cenatiempo and J. Frère (2001). Analysis of sigma(54)-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (Eil(Man)) is involved in sensitivity to a bacteriocin, mesentericin Y105. *Microbiology* **147**: 1575-80.
- Héchard, Y. and H. G. Sahl (2002). Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie* **84**: 545-57.
- Hill, C., P. D. Cotter, R. D. Sleator and C. G. M. Gahan (2002). Bacterial stress response in *Listeria monocytogenes*: jumping the hurdles imposed by minimal processing. *Int Dairy J* **12**: 273-283.

- Ho, J. L., K. N. Shands, G. Friedland, P. Eckind and D. W. Fraser (1986). An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. *Arch Intern Med* **146**: 520-4.
- Hof, H. (2001). *Listeria monocytogenes*: A causative agent of gastroenteritis? *Eur J Clin Microbiol Infect Dis* **20**: 369-373.
- Hof, H., R. Lampidis and J. Bensch (2000). Nosocomial *listeria* gastroenteritis in a newborn, confirmed by random amplification of polymorphic DNA. *Clin Microbiol Infect* **6**: 683-6.
- Jacquet, C., M. Doumith, J. I. Gordon, P. M. V. Martin, P. Cossart and M. Lecuit (2004). A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. *J Infect Dis* **189**: 2094-2100.
- Janda, J. M. and S. L. Abbott (1999). Unusual food-borne pathogens. *Listeria monocytogenes*, *Aeromonas*, *Plesiomonas*, and *Edwardsiella* species. *Clin Lab Med* **19**: 553-82.
- Jenkins, E. M., A. N. Njoku-Obi and E. W. Adams (1964). Purification of the soluble hemolysin of *Listeria monocytogenes*. *J Bacteriol* **88**: 418-424.
- Jensen, V. B., J. T. Harty and B. D. Jones (1998). Interactions of the invasive pathogens *Salmonella typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* with M cells and murine Peyer's patches. *Infect Immun* **66**: 3758-66.
- Jiménez-Díaz, R., R. M. Rios-Sánchez, M. Desmazeaud, J. L. Ruiz-Barba and J.-C. Piard (1993). Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Appl Environ Microbiol* **59**: 1416-1424.
- Johnsen, L., G. Fimland and J. Nissen-Meyer (2004). The C-terminal domain of pediocin-like antimicrobial peptides (class IIa bacteriocins) is involved in specific recognition of the C-terminal part of cognate immunity proteins and in determining the antimicrobial spectrum. *J Biol Chem*.
- Junttila, J. R., S. I. Niemela and J. Hirn (1988). Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *Listeria*. *J Appl Bacteriol* **65**: 321-7.
- Jurado, R. L., M. M. Farley, E. Pereira, R. C. Harvey, A. Schuchat, J. D. Wenger and D. S. Stephens (1993). Increased risk of meningitis and bacteremia due to *Listeria monocytogenes* in patients with human immunodeficiency virus infection. *Clin Infect Dis* **17**: 224-7.
- Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev* **12**: 39-85.

- Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon and P. Cossart (1992). *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. *Cell* **68**: 521-531.
- Kocks, C., R. Hellio, P. Gounon, H. Ohayon and P. Cossart (1993). Polarized distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. *J Cell Sci* **105** (Pt 3): 699-710.
- Kotrba, P., M. Inui and H. Yukawa (2001). Bacterial phosphotransferase system (PTS) in carbohydrate uptake and control of carbon metabolism. *J Biosci Bioeng* **92**: 502-517.
- Lawrence, L. M., J. Harvey and A. Gilmour (1993). Development of a random amplification of polymorphic DNA typing method for *Listeria monocytogenes*. *Appl Environ Microbiol* **59**: 3117-9.
- Leistner, L. (2000). Basic aspects of food preservation by hurdle technology. *Int J Food Microbiol* **55**: 181-6.
- Liang, P., L. Averboukh and A. B. Pardee (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic Acids Res* **21**: 3269-75.
- Lin, Y. D. and C. C. Chou (2004). Effect of heat shock on thermal tolerance and susceptibility of *Listeria monocytogenes* to other environmental stresses. *Food Microbiol* **21**: 605-610.
- Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, C. Salminen, D. W. Hird, M. L. Yonekura, P. Hayes, R. Weaver, A. Audurier, B. D. Plikaytis, S. L. Fannin, A. Kleks and C. V. Broome (1988). Epidemic listeriosis associated with Mexican-style cheese. *N. Engl. J. Med.* **319**: 823-828.
- Linnett, P. E. and J. L. Strominger (1973). Additional antibiotic inhibitors of peptidoglycan synthesis. *Antimicrob Agents Chemother* **4**: 231-6.
- Liu, D., M. L. Lawrence, A. J. Ainsworth and F. W. Austin (2005). Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. *FEMS Microbiol Lett* **243**: 373-8.
- Lou, Y. and A. E. Yousef (1997). Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. *Appl Environ Microbiol* **63**: 1252-5.

- Lowe, K. (2000). Recent advances in fluorescent differential display. Differential Display. R. A. Leslie and H. A. Robertson. New York, Oxford University Press: 5-33.
- Maftah, A., D. Renault, C. Vignoles, Y. Héchard, P. Bressollier, M. H. Ratinaud, Y. Cenatiempo and R. Julien (1993). Membrane permeabilization of *Listeria monocytogenes* and mitochondria by the bacteriocin mesentericin Y105. *J Bacteriol* **175**: 3232-5.
- Märki, F., E. Hänni, A. Fredenhagen and J. van Oostrum (1991). Mode of action of the lanthionine-containing peptide antibiotics duramycin, duramycin B and C, and cinnamycin as indirect inhibitors of phospholipase A2. *Biochem Pharmacol* **42**: 2027-35.
- Marquis, H., V. Doshi and D. A. Portnoy (1995). The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells. *Infect Immun* **63**: 4531-4.
- Martínez, B., A. Rodríguez and J. E. Suárez (2000). Lactococcin 972, a bacteriocin that inhibits septum formation in lactococci. *Microbiology* **146**: 949-55.
- Martínez, B., J. E. Suárez and A. Rodríguez (1996). Lactococcin 972: a homodimeric lactococcal bacteriocin whose primary target is not the plasma membrane. *Microbiology* **142**: 2393-2398.
- Matsuno, K. and A. L. Sonenshein (1999). Role of SpoVG in asymmetric septation in *Bacillus subtilis*. *J Bacteriol* **181**: 3392-401.
- McClure, P. J., T. A. Roberts and O. P.O. (1989). Comparison of the effects of sodium chloride, pH and temperature on the growth of *Listeria monocytogenes* on gradient plates and in liquid medium. *Lett Appl Microbiol* **9**: 95-99.
- McCormick, J. K., A. Poon, M. Sailer, Y. Gao, K. L. Roy, L. M. McMullen, J. C. Vederas, M. E. Stiles and M. J. Van Belkum (1998). Genetic characterization and heterologous expression of brochocin-C, an antitubulin, two-peptide bacteriocin produced by *Brochothrix campestris* ATCC 43754. *Appl Environ Microbiol* **64**: 4757-66.
- McEntire, J. C., G. M. Carman and T. J. Montville (2004). Increased ATPase activity is responsible for acid sensitivity of nisin-resistant *Listeria monocytogenes* ATCC 700302. *Appl Environ Microbiol* **70**: 2717-21.
- McLauchlin, J. (1990a). Distribution of serovars of *Listeria monocytogenes* isolated from different categories of patients with listeriosis. *Eur J Clin Microbiol Infect Dis* **9**: 210-3.

- McLauchlin, J. (1990b). Human listeriosis in Britain, 1967-85, a summary of 722 cases.
1. Listeriosis during pregnancy and in the newborn. *Epidemiol Infect* **104**: 181-9.
- McLauchlin, J. (1990c). Human listeriosis in Britain, 1967-85, a summary of 722 cases.
2. Listeriosis in non-pregnant individuals, a changing pattern of infection and seasonal incidence. *Epidemiol Infect* **104**: 191-201.
- McLauchlin, J. (1996). The relationship between *Listeria* and listeriosis. *Food Control* **7**: 187-193.
- McLauchlin, J. (1997). The pathogenicity of *Listeria monocytogenes*: a public health perspective. *Rev Med Microbiol* **8**: 1-14.
- McMahon, C. M., C. M. Byrne, J. J. Sheridan, D. A. McDowell, I. S. Blair and T. Hegarty (2000). The effect of culture growth phase on induction of the heat shock response, in *Yersinia enterocolitica* and *Listeria monocytogenes*. *J Appl Microbiol* **89**: 198-206.
- Medhurst, A. D., D. Chambers, J. Gray, J. B. Davis, J. A. Shale, I. Mason, P. Jenner and R. A. Newton (2000). Practical aspects of the experimental design for differential display of transcripts obtained from complex tissues. *Differential Display*. H. A. Robertson. New York, Oxford University Press: 35-66.
- Mengaud, J., H. Ohayon, P. Gounon, R. M. Mège and P. Cossart (1996). E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells. *Cell* **84**: 923-32.
- Modi, K. D., M. L. Chikindas and T. J. Montville (2000). Sensitivity of nisin-resistant *Listeria monocytogenes* to heat and the synergistic action of heat and nisin. *Lett Appl Microbiol* **30**: 249-53.
- Moll, G. N., W. N. Konings and A. J. Driessen (1999a). Bacteriocins: mechanism of membrane insertion and pore formation. *Antonie Van Leeuwenhoek* **76**: 185-98.
- Moll, G. N., E. van den Akker, H. H. Hauge, J. Nissen-Meyer, I. F. Nes, W. N. Konings and A. J. Driessen (1999b). Complementary and overlapping selectivity of the two-peptide bacteriocins plantaricin EF and JK. *J Bacteriol* **181**: 4848-52.
- Moorhead, S. M. and G. A. Dykes (2003). The role of the sigB gene in the general stress response of *Listeria monocytogenes* varies between a strain of serotype 1/2a and a strain of serotype 4c. *Curr Microbiol* **46**: 461-6.
- Murray, E. G. D., R. A. Webb and M. B. R. Swann (1926). A disease of rabbits characterized by large mononuclear leucocytosis, caused by a hitherto undescribed bacillus *Bacterium monocytogenes*. *J Pathol Bacteriol* **29**: 407-439.

- Nair, S., I. Derre, T. Msadek, O. Gaillot and P. Berche (2000). CtsR controls class III heat shock gene expression in the human pathogen *Listeria monocytogenes*. *Mol Microbiol* **35**: 800-11.
- Nair, S., C. Frehel, L. Nguyen, V. Escuyer and P. Berche (1999). ClpE, a novel member of the HSP100 family, is involved in cell division and virulence of *Listeria monocytogenes*. *Mol Microbiol* **31**: 185-96.
- Nes, I. F., D. B. Diep, L. S. Havarstein, M. B. Brurberg, V. Eijsink and H. Holo (1996). Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie Van Leeuwenhoek* **70**: 113-28.
- O'Driscoll, B., C. G. Gahan and C. Hill (1996). Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence. *Appl Environ Microbiol* **62**: 1693-8.
- Olson, A. (2003). Isolation and characterization of potentially pathogenic *Escherichia coli* present during beef production. Department of Agricultural, Food, and Nutritional Science. Edmonton, University of Alberta: 147.
- Periago, P. M., W. van Schaik, T. Abee and J. A. Wouters (2002). Identification of proteins involved in the heat stress response of *Bacillus cereus* ATCC 14579. *Appl Environ Microbiol* **68**: 3486-95.
- Piard, J.-C., P. M. Muriana, M. J. Desmazeaud and T. R. Klaenhammer (1992). Purification and partial characterization of lacticin 481, a lanthionine-containing bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CNRZ 481. *Appl Environ Microbiol* **58**: 279-284.
- Pron, B., C. Boumaila, F. Jaubert, S. Sarnacki, J. P. Monnet, P. Berche and J. L. Gaillard (1998). Comprehensive study of the intestinal stage of listeriosis in a rat ligated ileal loop system. *Infect Immun* **66**: 747-55.
- Quivey, R. G., Jr., R. Faustoferri, K. Monahan and R. Marquis (2000). Shifts in membrane fatty acid profiles associated with acid adaptation of *Streptococcus mutans*. *FEMS Microbiol Lett* **189**: 89-92.
- Racz, P., K. Tenner and E. Mero (1972). Experimental *Listeria* enteritis. I. An electron microscopic study of the epithelial phase in experimental listeria infection. *Lab Invest* **26**: 694-700.
- Ramage, C. P., J. C. Low, J. McLauchlin and W. Donachie (1999). Characterisation of *Listeria ivanovii* isolates from the UK using pulsed-field gel electrophoresis. *FEMS Microbiol Lett* **170**: 349-53.

- Ramnath, M., S. Arous, A. Gravesen, J. W. Hastings and Y. Héchard (2004). Expression of *mptC* of *Listeria monocytogenes* induces sensitivity to class IIa bacteriocins in *Lactococcus lactis*. *Microbiology* **150**: 2663-8.
- Ramnath, M., M. Beukes, K. Tamura and J. W. Hastings (2000). Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Appl Environ Microbiol* **66**: 3098-101.
- Riedo, F. X., R. W. Pinner, M. L. Tosca, M. L. Cartter, L. M. Graves, M. W. Reeves, R. E. Weaver, B. D. Plikaytis and C. V. Broome (1994). A point-source foodborne listeriosis outbreak: documented incubation period and possible mild illness. *J Infect Dis* **170**: 693-6.
- Robertson, J. B., M. Gocht, M. A. Marahiel and P. Zuber (1989). *AbrB*, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthesis gene. *Proc Natl Acad Sci U S A* **86**: 8457-61.
- Robichon, D., E. Gouin, M. Débarbouillé, P. Cossart, Y. Cenatiempo and Y. Héchard (1997). The *rpoN* (σ^{54}) gene from *Listeria monocytogenes* is involved in resistance to mesentericin Y105, an antibacterial peptide from *Leuconostoc mesenteroides*. *J Bacteriol* **179**: 7591-4.
- Rocourt, J. (1996). Risk factors for listeriosis. *Food Control* **7**: 195-202.
- Rosen, H., S. Gordon and R. J. North (1989). Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. Absence of monocytes at infective foci allows *Listeria* to multiply in nonphagocytic cells. *J Exp Med* **170**: 27-37.
- Rouquette, C., M. T. Ripio, E. Pellegrini, J. M. Bolla, R. I. Tascon, J. A. Vazquez-Boland and P. Berche (1996). Identification of a *ClpC* ATPase required for stress tolerance and in vivo survival of *Listeria monocytogenes*. *Mol Microbiol* **21**: 977-87.
- Sambrook, J. and D. W. Russel (2001). Protocol 1 Preparation of plasmid DNA by alkaline lysis with SDS: Miniprep. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press: 1.32-1.34.
- Schlech, W. F., 3rd, P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, A. W. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls and C. V. Broome (1983). Epidemic listeriosis--evidence for transmission by food. *N Engl J Med* **308**: 203-6.

- Sleator, R. D., G. A. Francis, D. O'Beirne, C. G. Gahan and C. Hill (2003). Betaine and carnitine uptake systems in *Listeria monocytogenes* affect growth and survival in foods and during infection. *J Appl Microbiol* **95**: 839-46.
- Sleator, R. D., C. G. Gahan and C. Hill (2001). Mutations in the listerial proB gene leading to proline overproduction: effects on salt tolerance and murine infection. *Appl Environ Microbiol* **67**: 4560-5.
- Sleator, R. D., C. G. M. Gahan, B. O'Driscoll and C. Hill (2000). Analysis of the role of betL in contributing to the growth and survival of *Listeria monocytogenes* LO28. *Int J Food Microbiol* **60**: 261-8.
- Tagg, J. R., A. S. Dajani and L. W. Wannamaker (1976). Bacteriocins of gram-positive bacteria. *Bacteriol Rev* **40**: 722-56.
- Tannock, G. W., A. Tilsala-Timisjarvi, S. Rodtong, J. Ng, K. Munro and T. Alatossava (1999). Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. *Appl Environ Microbiol* **65**: 4264-7.
- Tilney, L. G. and D. A. Portnoy (1989). Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *J Cell Biol* **109**: 1597-608.
- Toba, T., E. Yoshioka and T. Itoh (1991). Lacticin, a bacteriocin produced by *Lactobacillus delbrueckii* subsp. *lactis*. *Lett App Microbiol* **12**: 43-45.
- Vadyvaloo, V., S. Arous, A. Gravesen, Y. Héchard, R. Chauhan-Haubrock, J. W. Hastings and M. Rautenbach (2004a). Cell-surface alterations in class IIa bacteriocin-resistant *Listeria monocytogenes* strains. *Microbiology* **150**: 3025-33.
- Vadyvaloo, V., J. W. Hastings, M. J. van der Merwe and M. Rautenbach (2002). Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. *Appl Environ Microbiol* **68**: 5223-30.
- Vadyvaloo, V., J. L. Snoep, J. W. Hastings and M. Rautenbach (2004b). Physiological implications of class IIa bacteriocin resistance in *Listeria monocytogenes* strains. *Microbiology* **150**: 335-40.
- van Belkum, M. J., J. Kok, G. Venema, H. Holo, I. F. Nes, W. N. Konings and T. Abee (1991). The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. *J Bacteriol* **173**: 7934-41.

- van Belkum, M. J. and M. E. Stiles (2000). Nonantibiotic antibacterial peptides from lactic acid bacteria. *Nat Prod Rep* **17**: 323-35.
- van Schaik, W., C. G. Gahan and C. Hill (1999). Acid-adapted *Listeria monocytogenes* displays enhanced tolerance against the antibiotics nisin and lacticin 3147. *J Food Prot* **62**: 536-9.
- van Schaik, W., M. H. Tempelaars, J. A. Wouters, W. M. de Vos and T. Abee (2004). The alternative sigma factor sigmaB of *Bacillus cereus*: response to stress and role in heat adaptation. *J Bacteriol* **186**: 316-25.
- Vázquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Domínguez-Bernal, W. Goebel, B. González-Zorn, J. Wehland and J. Kreft (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* **14**: 584-640.
- Venema, K., T. Abee, A. J. Haandrikman, K. J. Leenhouts, J. Kok, W. N. Konings and G. Venema (1993). Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. *Appl Environ Microbiol* **59**: 1041-1048.
- Venema, K., R. E. Haverkort, T. Abee, A. J. Haandrikman, K. J. Leenhouts, L. de Leij, G. Venema and J. Kok (1994). Mode of action of LciA, the lactococcin A immunity protein. *Mol Microbiol* **14**: 521-32.
- Weeks, M. E., D. C. James, G. K. Robinson and C. M. Smales (2004). Global changes in gene expression observed at the transition from growth to stationary phase in *Listeria monocytogenes* ScottA batch culture. *Proteomics* **4**: 123-35.
- Weis, J. and H. P. Seeliger (1975). Incidence of *Listeria monocytogenes* in nature. *Appl Microbiol* **30**: 29-32.
- WHO/FAO (2004). Risk assessment of *Listeria monocytogenes* in ready-to-eat foods: Interpretative summary. Microbiological risk assessment series.
- Wiedmann, M., J. L. Bruce, C. Keating, A. E. Johnson, P. L. McDonough and C. A. Batt (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infect Immun* **65**: 2707-16.
- Wonderling, L. D., B. J. Wilkinson and D. O. Bayles (2004). The *htrA* (*degP*) gene of *Listeria monocytogenes* 10403S is essential for optimal growth under stress conditions. *Appl Environ Microbiol* **70**: 1935-43.

7 Appendix

DNA sequence of fragment JM1 and homology with known sequences

Sequence:

5' - GATGAGTCCTGACCGATAACACGAATGTCGTGAATAACAACTCACCGT
CAATCGTTATTGAAGAAATAGCTTTCATTCTCCCATCTGTCTCAACAGGTACG
CAGTCTACGAGACCAGA - 3'

NCBI blastn results:

gi|16409560|emb|AL591974.1| *Listeria monocytogenes* strain EGD, complete
genome, segment 2/12
Length = 160050

Score = 157 bits (79), Expect = 7e-36
Identities = 82/83 (98%)
Strand = Plus / Minus

Query: 14 cgataaacgaatgtcgtgaataacaaactcacgctcaatcgttattgaagaaatagctt 73
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
Sbjct: 205 cgatgacacgaatgtcgtgaataacaaactcacgctcaatcgttattgaagaaatagctt 146

```
Query: 74   tcattctcccatctgtctcaaca 96
           |||||
Sbjct: 145  tcattctcccatctgtctcaaca 123
```

```

FEATURES             Location/Qualifiers
     source            1..160050
                        /organism="Listeria monocytogenes"
                        /mol_type="genomic DNA"
                        /strain="EGD-e"
                        /db_xref="taxon:1639"
     gene            80..402
                        /gene="lmo0196"
     RBS             80..85
                        /gene="lmo0196"
     CDS            94..402
                        /gene="lmo0196"
                        /note="similar to B. subtilis SpoVG protein"
                        /codon_start=1
                        /transl_table=11
                        /protein_id="CAD00723.1"
                        /db_xref="GI:16409561"
                        /db_xref="GOA:Q8YAD5"
                        /db_xref="InterPro:IPR007170"
                        /db_xref="UniProt/TrEMBL:Q8YAD5"
                        /translation="MEITDVLRRLRVETDGRMKAISSITIDGEFVIHDIRVIDGNEGLF
VAMPSKRTPDGGEFRDIAHPINSGRTRAKIQEAVLAAYEVADEPAVNEESSADESIVEEN

```

DNA sequence of fragment JM2 and homology with known sequences

Sequence:

5' - ATGAGTCCTGACCGAAGTAAAGAGTCATGGAGGTAGAGCACTGTTTGAA
CTAGGGGCCCTTCTCGGGTTACCGAATTCAGATAAACTCCGAATGCCATGTAC
TTATACTCGGGAGTCAGACTGCGAGTGATAAGATCCGTAGTCGGGTACGCAG
TCTACGAGACCAA - 3'

NCBI blastn results:

>gi|44089|emb|X64533.1|LM23SRDNA L.monocytogenes 23S ribosomal DNA
Length = 2928

Score = 248 bits (125), Expect = 4e-63
Identities = 128/129 (99%)
Strand = Plus / Plus

Query: 17 gtaaagagtcatggaggtagagcactgtttgaactaggggcccttctcgggttaccgaat 76
|||||
Sbjct: 890 gtaaagagtcatggaggtagagcactgtttggactaggggcccttctcgggttaccgaat 949

Query: 77 tcagataaaactccgaatgccatgtacttatactcgggagtcagactgcgagtgataagat 136
|||||
Sbjct: 950 tcagataaaactccgaatgccatgtacttatactcgggagtcagactgcgagtgataagat 1009

Query: 137 ccgtagtcg 145
|||||
Sbjct: 1010 ccgtagtcg 1018

DNA sequence of fragment JM3 and homology with known sequences

Sequence:

5' - ACTGGTCTCGTAGACTGCGTACCCCCGAGTATAAGTACATGGCATTTCGGA
GTTTATCTGAATTCGGTAACCCGAGAAGGGCCCCTAGCTCCAAAGCAGTGCT
CTACCTCCATGACTCTTTACTTCGGTCAGGACTCATA - 3'

NCBI blastn results:

gi|44089|emb|X64533.1|LM23SRDNA L.monocytogenes 23S ribosomal DNA
Length = 2928

Score = 165 bits (83), Expect = 3e-38
Identities = 97/99 (97%), Gaps = 2/99 (2%)
Strand = Plus / Minus

Query: 24 cccgagtataagtacatggcattcggagtttatctgaattcggtaacccgagaagggccc 83
|||||
Sbjct: 986 cccgagtataagtacatggcattcggagtttatctgaattcggtaacccgagaagggccc 927

Query: 84 ctagctccaaagcagtgcctctacctccatgactctttac 122
||| |||||
Sbjct: 926 ctag-tccaaa-cagtgcctctacctccatgactctttac 890

DNA sequence of fragment JM4 and homology with known sequences

Sequence:

5' - ATGAGTCCTGACCGACAGGGAAACAGCCCAGACCACCAGTTAAGGTCCC
CAAATATATGTTAAGTGGAAAAGGATGTGGGGTTGCTTAGACAACCAGGATG
TTGGCTTAGAAGCAGCCACCATTGAAAGAGTGCGTAATAGCTCACTGGTCGG
GTACGCAGTCTACGAGACCAGA - 3'

NCBI blastn results:

>gi|44089|emb|X64533.1|LM23SRDNA L.monocytogenes 23S ribosomal DNA
Length = 2928

Score = 270 bits (136), Expect = 1e-69
Identities = 136/136 (100%)
Strand = Plus / Plus

Query: 17 agggaaacagcccagaccaccagttaaggtccccaatatatgttaagtggaaaaggatg 76
|||||
Sbjct: 1021 agggaaacagcccagaccaccagttaaggtccccaatatatgttaagtggaaaaggatg 1080

Query: 77 tgggggttgcttagacaaccaggatggttgcttagaagcagccaccattgaaagagtgcgt 136
|||||
Sbjct: 1081 tgggggttgcttagacaaccaggatggttgcttagaagcagccaccattgaaagagtgcgt 1140

Query: 137 aatagctcactgggtcg 152
|||||
Sbjct: 1141 aatagctcactgggtcg 1156